台灣農業研究

JOURNAL OF TAIWAN AGRICULTURAL RESEARCH

Vol.74 No.4 2025

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ISSN 2790-086X
9 772790 086007

Published by Taiwan Agricultural Research Institute, Ministry of Agriculture

台灣農業研究 JOURNAL OF TAIWAN AGRICULTURAL RESEARCH Vol.74 No.4 2025

ISSN 2790-086X
DOI:10.6156/JTAR

台灣農業研究

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農業部農業試驗所出版

台灣農業研究 (J. Taiwan Agric. Res.) 74(4):359–376 (2025)
https://doi.org/10.6156/JTAR.202512_74(4).0001

Feature Article

Top-Down and Bottom-Up Approaches in Phytoplasma Research: Advancing Pathogenesis, Taxonomy, and Diagnostics

Wei Wei[1,*]

Abstract

Wei, W. 2025. Top-down and bottom-up approaches in phytoplasma research: Advancing pathogenesis, taxonomy, and diagnostics. J. Taiwan Agric. Res. 74(4):359–376.

Phytoplasmas are minute, cell wall-less bacteria responsible for devastating plant diseases, leading to significant economic loss in agriculture. This article explores recent advancements in phytoplasma research, focusing on pathogenesis, taxonomy, and diagnostics through top-down and bottom-up approaches. Top-down multi-omics studies have provided a system-level understanding of phytoplasma-induced disruptions, particularly in sugar metabolism and hormone signaling, revealing their extensive impact on plant physiology. Complementing this, bottom-up strategies have dissected molecular interactions, elucidating how phytoplasmas derail meristem fate, modulate plant growth patterns, alter plant architecture, and induce characteristic symptoms. Advances in taxonomy and classification have improved species differentiation, integrating 16S rRNA sequencing, multilocus sequence typing (MLST), and whole-genome sequencing (WGS), with database-guided tools refining classification accuracy. The development of cutting-edge diagnostic technologies, such as clustered regularly interspaced short palindromic repeats-based (CRISPR-based) detection, has significantly enhanced the sensitivity, specificity, and efficiency of phytoplasma identification and surveillance. Additionally, the integration of big data analytics and AI-driven models has pioneered image-based symptom recognition, supporting disease surveillance and monitoring.

Key words: Meristem fate derailment, *i*PhyClassifier, Artificial intelligence (AI), Omics, Floral transition.

INTRODUCTION

Phytoplasmas are small bacteria that infect a wide range of plants, causing severe economic loss worldwide. Residing in plant phloem tissues and being adapted to the nutrient-rich environment, phytoplasmas have gone through reductive evolution, as manifested by the lack of many genes that are involved in metabolic pathways essential to free-living organisms (Oshima *et al*. 2004; Kube *et al*. 2012; Tan *et al*. 2021). In nature, phytoplasmas are transmitted by phloem-feeding insect vectors, primarily leafhoppers, planthoppers, and psyllids, which acquire the pathogen while feeding infected plants and spreading it to healthy ones. This vector-mediated transmission enables phytoplasmas to persist in ecosystems and infect a wide range of plant species, often leading to devastating outbreaks in both agricultural and natural environments (Weintraub & Beanland 2006). Phytoplasmas cannot be cultured *in vitro* (Wei & Zhao 2022), presenting a significant challenge for research, diagnostics, and disease management.

Received: June 16, 2025; Accepted: August 13, 2025.
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Phytoplasma-infected plants exhibit a diverse array of symptoms, including virescence (greening of floral organs), phyllody (conversion of floral structures into leaf-like formations), witches'-broom (WB) (excessive shoot proliferation), yellowing, and stunted growth (Wei *et al.* 2013, 2022). These physiological disruptions can severely impact plant development and productivity, leading to economically significant diseases such as grapevine yellows, date palm dieback, and apple proliferation (Seemüller & Schneider 2004; Belli *et al.* 2010; Gurr *et al.* 2016).

Beyond their detrimental effects, phytoplasmas can induce unusual yet visually striking morphological changes, which are sometimes considered beneficial in horticultural settings. For example, phytoplasma-associated poinsettias exhibit a branching phenotype, which is more attractive in aesthetics (Lee *et al.* 1997). The ability of these pathogens to manipulate plant development raises a fundamental question: What molecular and cellular mechanisms drive these alterations in plant morphology? Understanding these mechanisms is essential for unraveling phytoplasma-host interactions and developing effective disease control strategies.

A systematic and integrative research approach is necessary to investigate the complexity of phytoplasma-induced morphological changes. This involves top-down and bottom-up methodologies (Shahzad & Loor 2012). The top-down approach, often applied through high-throughput omics studies, provides a broad overview of host responses at the genomic, transcriptomic, proteomic, and metabolomic levels (Tan *et al.* 2021, 2025). This comprehensive perspective helps identify global changes in gene expression and biochemical pathways that occur upon phytoplasma infection. In contrast, the bottom-up approach focuses on specific molecular players, including key regulatory genes, proteins, and signaling molecules. Research using this approach has identified meristem-regulating genes and floral organ identity genes that undergo disruption upon infection, leading to developmental reprogramming (MacLean *et al.* 2011; Sugio *et al.* 2011; Wei *et al.* 2013, 2019, 2022). Additionally, several phytoplasma-derived effector proteins have been identified as key factors in manipulating plant growth by targeting host regulatory pathways (Hoshi *et al.* 2009; MacLean *et al.* 2011; Sugio *et al.* 2011; Maejima *et al.* 2014; Huang *et al.* 2021).

These complementary approaches enhance the understanding of how phytoplasmas alter plant architecture and provide valuable insights into plant-pathogen interactions. By deciphering these molecular mechanisms, future strategies can be developed to mitigate the negative impacts of phytoplasma diseases while exploring potential applications for controlled morphological modifications in agriculture and horticulture.

Phytoplasma taxonomy has transitioned from early misclassification to a molecular-based framework, addressing challenges associated with their unculturable nature (Wei & Zhao 2022). The *Candidatus* Phytoplasma designation, along with whole-genome sequencing (WGS), average nucleotide identity (ANI), and multilocus sequence typing (MLST), has refined species delineation and classification (Bertaccini *et al.* 2022; Wei & Zhao 2022). The *i*PhyClassifier database further standardizes strain identification, automating classification and expanding reference datasets (Zhao *et al.* 2009; Wei & Zhao 2022). Advances in molecular diagnostics, including polymerase chain reaction-based (PCR-based) methods (Lee *et al.* 1993), loop-mediated isothermal amplification (LAMP) (Dickinson 2015), and clustered regularly interspaced short palindromic repeats-based (CRISPR-based) detection (Wheatley *et al.* 2022), have improved disease surveillance and management.

AI and data science are introducing new approaches to phytoplasma research, particularly in disease monitoring and classification. AI-driven image analysis using convolutional neural networks (CNNs) shows promise in assisting disease detection, while databases such as *i*PhyDSDB and the Phytoplasma Disease and Classification Database enhance disease rec-

ognition and classification (Wei *et al*. 2024a, 2024b). Big data analytics are also improving the understanding of disease patterns, aiding more informed management strategies. Ongoing research into phytoplasma biology, taxonomy, and disease monitoring, combined with advancements in molecular diagnostics, AI, and big data analytics, may further enhance disease detection and management in agriculture.

PHYTOPLASMA-INDUCED ALTERATIONS OF PLANT GROWTH PATTERNS AND ARCHITECTURE

Sexual reproduction is a key process in the life cycle of seed plants, occurring in three main phases: flowering, fruit formation, and seed dispersal, followed by germination (Lord & Russell 2002). The transition to flowering marks the shift from vegetative to reproductive growth, regulated by genetic programs and environmental cues such as light, temperature, and hormones. During this stage, the shoot apical meristem transforms, with the vegetative meristem (VM) converting into an inflorescence meristem (IM), which generates floral meristems (FMs). These FMs develop into sepals, petals, stamens (male reproductive organs), and carpels (female reproductive organs containing ovaries and ovules) (Kater *et al*. 2006). Once carpels form, FMs cease further meristematic activity.

Pollination, facilitated by wind, water, or pollinators, transfers pollen from the stamen to the carpel, where fertilization occurs, initiating seed and fruit development (Seymour *et al*. 2013). The fruit protects seeds and aids in their dispersal through various mechanisms. Dormant seeds remain inactive until favorable conditions trigger germination, reactivating metabolic processes and stimulating root and shoot apical meristems for new growth (Koornneef *et al*. 2002). From flowering to germination, this cycle is tightly regulated by genetic pathways and environmental factors, ensuring reproductive success and species survival (De Kroon *et al*. 2005; Barthélémy & Caraglio 2007). In healthy plants, once the floral transition is complete, the formation of flowers, fruits, and seeds follows a predetermined, irreversible process. However, in plants infected by phytoplasmas, this natural progression is disrupted, leading to distinct abnormalities such as virescence, phyllody, and arrested inflorescence, leading to a significant reduction in fruit set and seed formation (Wei *et al*. 2013, 2019).

A bottom-up approach was employed to explore the mechanism underlying these morphological alterations, focusing on Columbia Basin potato purple top (PPT) phytoplasma and its alternate host, the tomato (the model pathogen-host pair our group is utilizing for interaction studies).

Small seedling graft inoculation

The experimental plants were established using a graft inoculation technique. PPT phytoplasma was introduced into small tomato seedlings at the four-leaf stage (rootstock, Wei *et al*. 2013, 2019). Infected shoots displaying WB symptoms were used for the phytoplasma inocula (scions). This small-seedling grafting method allowed for a controlled infection process, providing a clear timeline for symptom progression and enabling researchers to observe the sequential development of phytoplasma-induced symptoms. The study challenged the previous assumption that phytoplasmas cause unique, host-specific symptoms. Instead, the findings demonstrated that a single phytoplasma strain can induce distinct symptoms depending on the meristematic stage it infects, highlighting the critical role of meristem fate in determining the nature of phytoplasma-induced abnormalities.

Phytoplasma infection-induced meristematic derailment

Phytoplasma infections exert their effects by disrupting meristems responsible for cell division and differentiation in plants. The specific developmental outcome of infection depends on whether the vegetative meristem, inflores-

cence meristem, floral meristem, or flower organ primordia are affected (Fig. 1, Wei *et al.* 2013). The floral transition follows a sequential shift in meristematic fate, progressing from the vegetative meristem to the inflorescence meristem, then to the floral meristem, and finally to flower organ primordia formation. However, when phytoplasmas infect plants, the disruption initially affects the later stages of floral transition, as the infection occurs after the process has already begun, primarily targeting the floral meristem and flower organ primordia. As symptoms progress, the infection gradually extends to earlier stages, including the inflorescence meristem and the vegetative meristem, leading to broader developmental abnormalities (Wei *et al.* 2013, 2019).

In a normal flowering tomato line, 28 d post inoculation (dpi), PPT phytoplasma infection directly affects the floral meristem. The development of flowers is prematurely terminated, leading to the formation of flattened floral meristems. This results in the big bud (BB) phenotype, where sepals enlarge abnormally, and petals, stamens, and carpels are underdeveloped and fail to develop into fully formed flowers (Fig. 1A).

At 45 dpi, PPT phytoplasma infection affects the inflorescence meristem, arresting the initiation of floral meristems. Instead of forming individual flowers, the plant continuously produces repetitive inflorescence meristems, resulting in a cauliflower-like inflorescence structure (CLI, Fig. 1B). This disruption suggests that phytoplasma alters the delicate regulatory balance between meristem maintenance

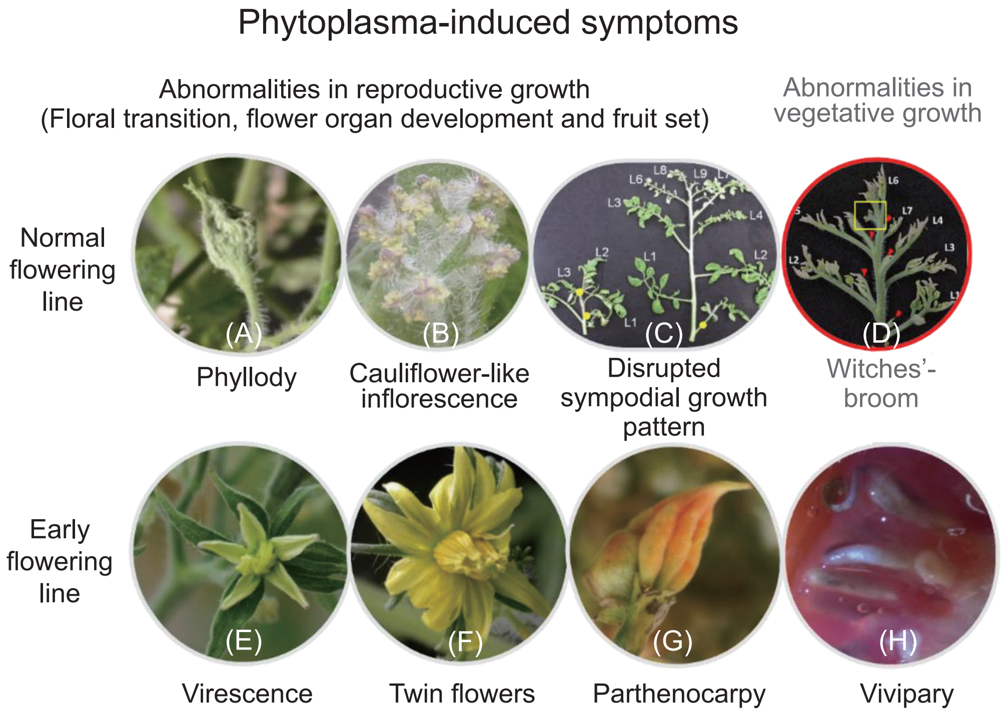

Fig. 1. Symptoms of potato purple top (PPT) phytoplasma infection in tomato plants showing abnormalities in vegetative and reproductive growth. (A–C) Abnormalities in reproductive growth in normal flowering tomato line (flower after 10–12 leaves). (A) Big bud (BB) or phyllody; (B) cauliflower-like inflorescence (CLI); (C) disrupted sympodial growth pattern (DSGP); (D) PPT phytoplasma-induced alterations in vegetative growth. Witches'-broom (WB) is characterized by dense clustering of shoots due to abnormal branching. (E–H) Abnormalities in reproductive growth in early flowering tomato line (flower after 3–5 leaves, E–H). (E) virescence; (F) twin flowers; (G) parthenocarpy; (H) vivipary.

and differentiation, preventing proper floral development.

When PPT phytoplasma infection targets the vegetative meristem, the transition from vegetative to reproductive growth is delayed, resulting in an extended vegetative phase with the production of excess leaves. Instead of three leaves per sympodium unit, more leaves are produced in PPT phytoplasma-infected plants. This phenomenon is called "disrupted sympodial growth pattern" (DSPG, Fig. 1C).

An early flowering tomato line, which overexpresses the Single Flower Truss (SFT) gene and flowers after producing three to five leaves (Lifschitz *et al*. 2006; Shalit *et al*. 2009), is also used for PPT phytoplasma infection. SFT, the tomato ortholog of Flowering Locus T (FT) in *Arabidopsis thaliana*, encodes florigen, a mobile signaling molecule that promotes flowering. By performing graft inoculation at the four-leaf stage combined with an early flowering line (35S: SFT), this setup provided an extended time window for phytoplasma-plant interactions, particularly during critical developmental phases of floral transition. As a result, more developmental abnormalities in flower, fruit, and seed formation are observed (Fig. 1E–G). Some plants develop virescence, where floral organs, especially petals, turn green (Fig. 1E), and twin flowers, where flower organs are duplicated (Fig. 1F). Another common abnormality is parthenocarpy, where fruits develop without fertilization (Fig. 1G). Additionally, very few plants set fruit, and some exhibit vivipary, where seeds begin germinating while still inside the fruit (Fig. 1H). This phenomenon indicates that phytoplasma infection disrupts seed dormancy mechanisms, possibly by altering abscisic acid (ABA) levels, which generally prevent premature germination. As symptoms progress, phytoplasma infection can primarily affect the early stages of floral transition; therefore, virescence, twin flowers, parthenocarpy, and vivipary are no longer observed; instead, like the normal flowering line, only BB and CLI remain detectable.

Gene regulation and functional studies

In phytoplasma-infected plants, genes involved in meristem maintenance and transition, including *SFT*, *FA*, and *AN*, as well as floral organ identity genes (*MC*, LePI, *TAP3*, *TAG*, and *TM5*) and those regulating floral organ primordia development, were found to be misregulated. These observations were made using gain- and loss-of-function tomato lines to dissect the regulatory impacts of infection. The data indicated that phytoplasma infection leads to the downregulation of inflorescence and floral meristem identity genes while simultaneously upregulating genes involved in meristem reiteration, explaining the prolonged vegetative growth, loss of floral determinacy, and floral malformations observed in infected plants (Wei *et al*. 2013, 2019).

Based on these findings, a working model was developed to explain how phytoplasma infection disrupts plant meristem function, leading to diverse developmental abnormalities. This model suggests that phytoplasma manipulates gene regulatory networks in three main ways: delaying floral transition by suppressing flowering genes, arresting floral meristem initiation by disrupting meristem maintenance pathways, and altering floral organ identity by misregulating homeotic gene expression (Wei *et al*. 2013, 2019).

PHYTOPLASMA-INDUCED ALTERATION IN VEGETATIVE GROWTH (WITCHES'-BROOM)

In addition to the symptoms tied to plant reproductive growth, phytoplasma-induced symptoms include a typical abnormality called "witches'-broom" (WB, Fig. 1D), which specifically affects the plant's vegetative growth. This branching arises from each leaf axil and further develops axillary buds, resulting in an excessive, broom-like appearance (Wei *et al*. 2013, 2022). While shoot branching is observed in plants infected by viruses, viroids, and fungi (Satoh *et al*. 2013; Kaur & Kaur 2018; Bao *et al*. 2019; de Haro *et al*. 2019), phytoplasma-in-

duced branching tends to be more severe. The underlying cause of this abnormal branching is suspected to be the disruption of plant sugar metabolism and hormonal balance due to the pathogen's presence.

Our top-down omics findings also indicate that phytoplasma infection leads to significant alterations in the sugar metabolism of host plants (Details, please refer to the next section). To elucidate the mechanism underlying the formation of WB, the bottom-up approach focusing on starch and sucrose was employed. A significant starch buildup was observed in PPT phytoplasma-infected leaf samples using Lugol's iodine staining (Wei *et al*. 2022). Under transmission electron microscopy (TEM), irregular and swollen starch granules were also found. This TEM image showed that the swelling starch granule occupied almost all the chloroplast, leading to damage to the chloroplast. This phytoplasma-induced blockage of starch breakdown triggers autophagy to degrade the injured chloroplasts (Wei *et al*. 2022; Inaba *et al*. 2023a).

The sucrose content in different plant tissues was measured, and the distribution of auxins and cytokinins was visualized using the reporter tomato lines, including DR5 (direct repeat 5)::GUS (β-glucuronidase) and ARR5 (arabidopsis response regulator 5)::GUS lines (Wei *et al*. 2022). The expression profile of genes involved in lateral bud initiation and outgrowth was assessed. The sucrose level was higher in source leaves and leaf axils and lower in stem and root (Wei *et al*. 2022). This indicates phytoplasma infection disrupts phloem translocation of sucrose, and this is caused by excessive callose deposition (Fig. 2). Aniline blue staining revealed that more callose was found in infected phloem compared to healthy phloem (Fig. 2A and B). Using TEM, it is evident that callose depositions sealed the sieve pores of infected plants (Fig. 2C and D). Our results showed that

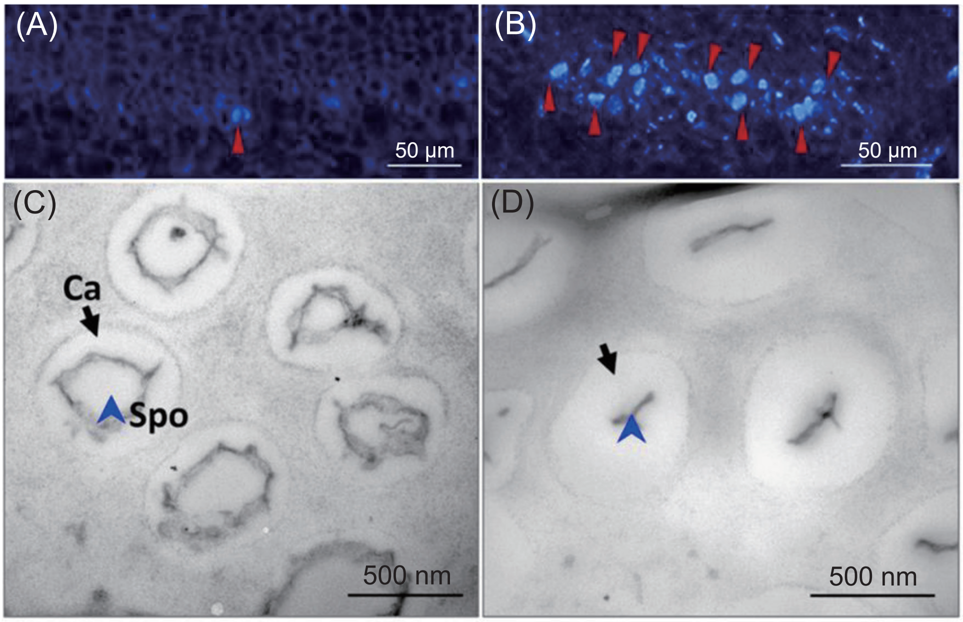

Fig. 2. Callose deposition in the tomato plants induced by potato purple top (PPT) phytoplasma. (A, B) Aniline blue staining of cross-stem sections of (A) mock control and (B) infected plants. Red triangles show the phloem's callose deposition (bright blue fluorescence). Scale bar = 50 μm. (C, D) Transmission electron microscopy (TEM) analysis revealed excessive callose deposition in the sieve plates of tomato plants induced by PPT phytoplasma. Blue arrowheads point to the sieve plate and sieve pore (Spo), respectively. Black arrows indicate callose (Ca) deposition. Scale bar = 500 nm. This figure is attributed to https://doi.org/10.3390/ijms23031810. Reproduced according to the terms of the Creative Commons Attribution License.

sucrose accumulation in leaf axils triggers the initiation of axillary buds. This process is complemented by the involvement of cytokinins, as evidenced by their distinct distribution in leaf axils (Fig. 3, Wei *et al*. 2022).

Moreover, the findings indicate that phytoplasma infection leads to early leaf senescence, and the marker genes that induce leaf early senescence were significantly upregulated. Phytoplasma-induced blockage of starch breakdown down-regulated expression of genes involved in gibberellin synthesis. Gibberellin is a well-known plant hormone that contributes to plant height. This is why the stunted growth was observed in phytoplasma-infected plants. Expression patterns of genes associated with axillary bud outgrowth, such as *BRC1* and *SPLs*, change infection, contributing to shoot branching (Wei *et al*. 2022).

Based on these findings, we proposed a model (Fig. 4), which demonstrates that phytoplasma-induced WB symptom is driven by a multitude of mechanisms, including the blockage of starch breakdown, chloroplast degradation, premature leaf senescence, sucrose reallocation, and cytokinin redistribution. As a result, infected plants exhibit leaf chlorosis, reduced leaf size (little leaf), and stunted growth. Furthermore, phytoplasma infection disrupts sugar metabolism and interferes with sucrose transport, causing sucrose to be redirected to leaf axils. This redistribution coincides with an increased cytokinin concentration, promoting axillary bud initiation. The continuous cycle of axillary bud formation and excessive outgrowth, coupled with accelerated leaf senescence, ultimately manifests as WB symptoms.

"OMICS" STUDIES ON PHYTOPLASMAS AND INFECTED PLANTS

The top-down approach was employed to study interactions between phytoplasmas and plants, including widely used genomics, transcriptomics, proteomics, and metabolomics (Tan *et al*. 2021), as well as additional derivative omics such as ubiquitomics, lipidomics, and volatilomics (Inaba *et al*. 2023b; Ivanauskas *et al*. 2023).

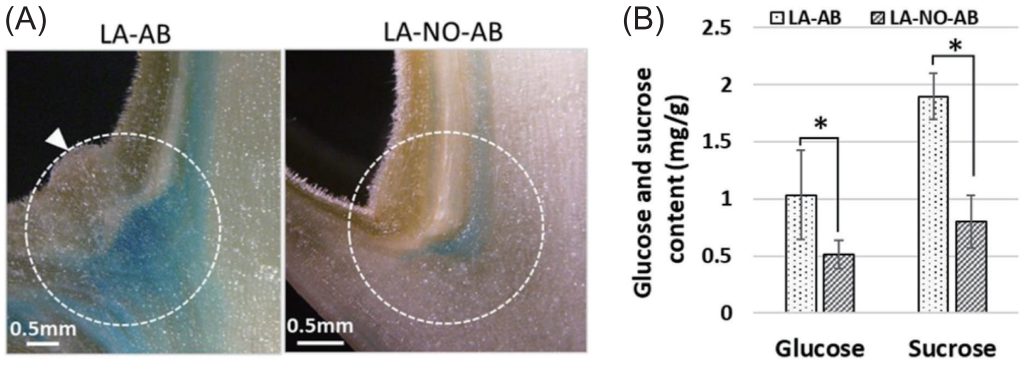

Fig. 3. Distribution of cytokinin-responsive signal and sugar content in leaf axils of ARR5 (arabidopsis response regulator 5)::GUS (β-glucuronidase) cytokinin reporter tomato plants infected with potato purple top (PPT) phytoplasma. (A) Cytokinin activity, as indicated by GUS staining, was analyzed in leaf axils of ARR5::GUS reporter tomato plants infected with PPT phytoplasma. Intense GUS staining, reflecting a high level of cytokinin, was observed in leaf axils where a new axillary bud had been initiated (LA-AB, left panel). In contrast, light GUS staining, indicating a lower cytokinin signal, was detected in leaf axils where no axillary bud developed (LA-NO-AB, right panel). The newly initiated bud is marked by a white triangle. Leaf axils (LA-ABs and LA-NO-ABs) highlighted with white circles were collected for sugar content analysis. Scale bar = 0.5 mm. (B) Glucose and sucrose levels were measured in the sampled leaf axils of PPT phytoplasma-infected plants. Significantly higher glucose and sucrose contents were detected in LA-ABs compared to LA-NO-ABs ($^{*}P < 0.01$), suggesting a correlation between cytokinin accumulation, sugar availability, and axillary bud initiation. This figure is attributed to https://doi.org/10.3390/ijms23031810. Reproduced according to the terms of the Creative Commons Attribution License.

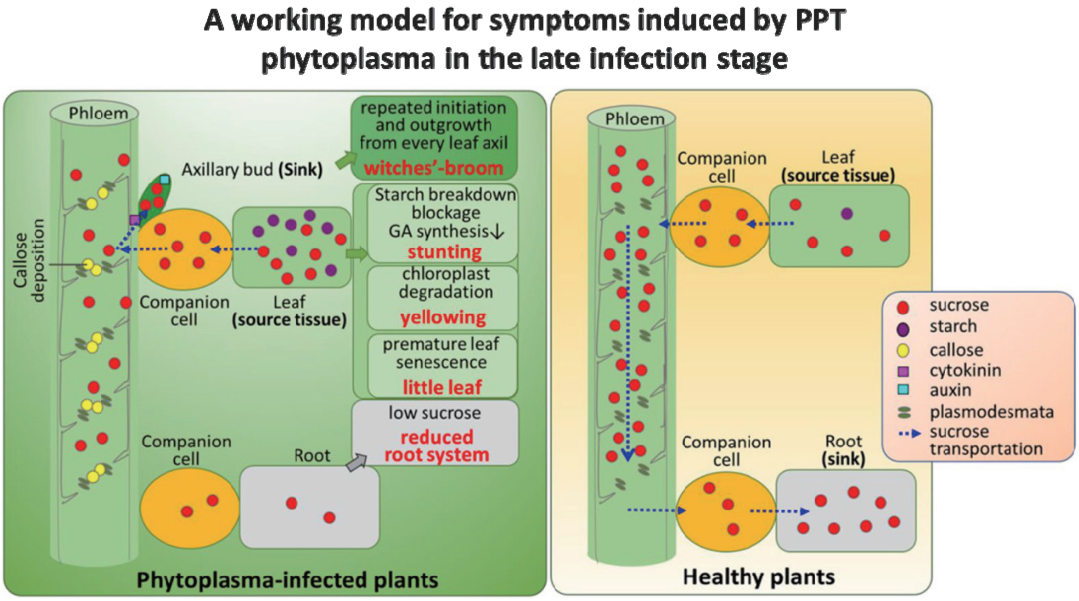

Fig. 4. A proposed working model for symptoms induced by potato purple top (PPT) phytoplasma in the late infection stage. In this model, PPT phytoplasma infection disrupts starch breakdown, leading to the degradation of damaged chloroplasts and premature leaf senescence. As a result, symptoms such as little leaf and leaf chlorosis emerge. The blockage of starch breakdown also suppresses gibberellin (GA) synthesis, restricting plant growth and reducing height. Additionally, PPT phytoplasma infection causes excessive callose deposition, reducing sieve pore size and impeding sucrose translocation through the phloem. This leads to sucrose accumulation in leaf axils, which, in turn, triggers axillary bud initiation. Cytokinin distribution in leaf axils contributes to this process, while auxin promotes the elongation of the axillary buds. Repetitive axillary bud initiation and outgrowth lead to witches'-broom formation. This figure is attributed to https://doi.org/10.3390/ijms23031810. Reproduced according to the terms of the Creative Commons Attribution License.

Genomic characteristics of phytoplasmas

Phytoplasma genomes are small and adenine-thymine-rich (AT-rich), with sizes ranging from 530 to 1,200 kb (Tan *et al*. 2021; Wei & Zhao 2022). Their reduced genome size results from extensive gene loss, a characteristic common among obligate intracellular pathogens. A defining feature of phytoplasma genomes is the presence of clustered, multiple-copy genes, forming a distinctive genomic architecture known as sequence-variable mosaics (SVMs) (Jomantiene *et al*. 2007). These structures are highly dynamic, repetitive sequences that exhibit high variability across different phytoplasma strains. The presence of these mosaics suggests an evolutionary mechanism that allows phytoplasmas to maintain genetic diversity despite their reduced genome size. This flexibility may play a critical role in their ability to adapt to different hosts, evade plant immune responses, and manipulate host physiology. These variable genomic regions have also been described as potential mobile units (PMUs) (Bai *et al*. 2006) or mobile unit genes (MUGs) (Arashida *et al*. 2008). The terminology reflects their suspected role in genomic plasticity, where mobile genetic elements facilitate genetic recombination, allowing phytoplasmas to acquire or modify new functions. These mobile units may contribute to the horizontal exchange of genes among phytoplasma strains, promoting adaptability in response to environmental pressures and interactions with diverse host species.

The origin of these mobile elements remained a mystery until their relationship to prophages was discovered (Wei *et al*. 2008a).

This finding linked the presence of SVMs and PMUs to bacteriophage-derived genetic material, suggesting that horizontal gene transfer via phage activity has significantly shaped phytoplasma genomes. Despite variations in genome sizes, after removing the prophage sequence, the genomes of all phytoplasmas were similar in size, around 550 to 580 kb, akin to the circular chromosome of *Mycoplasma genitalium*, known for its minimal gene set for cellular life (Zhao *et al*. 2014). No SVMs were observed in acholeplasmas, spiroplasmas, or mycoplasmas, which share a close phylogenetic relationship with phytoplasmas. However, all phytoplasmas possess SVMs. This discovery implies that phage-mediated recombination has played a fundamental role in the evolution of phytoplasmas, introducing new genetic material that may enhance virulence, host adaptation, and transmission efficiency. Phytoplasmas likely acquired these mobile elements through repeated cycles of phage infection, allowing them to incorporate and retain beneficial genetic fragments over time.

Another common trait within phytoplasma genomes is the absence of various genes related to metabolic pathways, including the tricarboxylic acid cycle, pentose phosphate pathway, sterol biosynthesis, fatty acid biosynthesis, *de novo* nucleotide synthesis, and biosynthesis of most amino acids (Oshima *et al*. 2004). However, phytoplasma genomes harbor multiple copies of transporter-related genes, such as ABC transporters, that can import nutrients into the cell. This indicates that phytoplasmas are highly dependent on metabolites imported from the hosts for their own growth and infection (Oshima *et al*. 2004).

Phytoplasma infection-induced metabolic reprogramming in plants

Phytoplasma infection induces significant metabolic reprogramming in host plants, leading to extensive alterations in primary and secondary metabolic pathways. By utilizing sweet cherry trees and sweet cherry virescence (SCV) phytoplasma, the metabolomics study identified 676 metabolites, with 187 differentially expressed metabolites. The majority of these belonged to categories such as carbohydrates, fatty acids/lipids, amino acids, and flavonoids, indicating a broad metabolic shift in response to infection. These findings suggest that phytoplasma systematically manipulates host metabolism to optimize conditions for its survival and proliferation (Tan *et al*. 2021).

One observation was the upregulation of glycolysis and pentose phosphate pathway (PPP) activity, which provides the energy and building blocks necessary for phytoplasma growth and replication. The infection-induced increase in glucose-6-phosphate, sedoheptulose 7-phosphate, and maltotetraose suggests a breakdown of carbohydrates (Tan *et al*. 2021). By promoting these pathways in the plant, phytoplasma ensures a steady influx of energy-rich compounds and metabolic intermediates, which are likely to sustain its proliferation.

Increased accumulation of several key sugars, such as D-glucose, D-glucose-6-phosphate, and D-sedoheptulose-7-phosphate, indicates a shift in source-to-sink relationships within the plant, potentially redistributing resources to favor the pathogen's needs. This metabolic reprogramming is consistent with previous transcriptomic studies showing upregulation of genes related to sugar transport and metabolism in phytoplasma-infected plants (Tan *et al*. 2019). Furthermore, the detection of melezitose, a sugar known to attract insect vectors, suggests that phytoplasma infection may also indirectly facilitate its own spread by modifying plant metabolism to enhance insect feeding behavior.

Transcriptomic insights into phytoplasma-induced symptoms (little leaf formation within WB structures)

The transcriptomic changes underlying the formation of little leaves within WB structures in sweet cherry trees infected by Sweet Cherry Virescence (SCV) phytoplasma were investigated. This research focuses on how phytoplasma infection alters plant growth, particularly in terms of leaf size reduction and premature se-

nescence (Tan *et al*. 2025). Morphological analysis revealed that infected leaves were smaller, yellowed, and irregularly shaped, with reduced palisade cell numbers and phloem lumen size, impairing expansion and sugar transport. Hormonal imbalances, including increased abscisic acid (ABA) and jasmonic acid (JA) but reduced cytokinins, contributed to growth inhibition and premature senescence.

Transcriptomic analysis revealed extensive changes in gene expression, particularly in pathways involved in ribosome biogenesis, DNA replication, and cell cycle progression. Many genes related to ribosome formation and protein synthesis were significantly downregulated, leading to an overall suppression of cell division. The downregulation of key cyclin genes (CYCD3; 1 and CYCD6; 1) suggests that phytoplasma infection causes a cell cycle arrest at the G1 phase, preventing proper leaf expansion and growth. Despite these disruptions, genes involved in photosynthesis and sugar metabolism were upregulated, likely as a compensatory response to the plant's energy deficit. However, this increased gene expression did not translate into higher sugar availability, as transport and utilization remained impaired (Tan *et al*. 2025).

Further analysis indicated that little leaf formation might be associated with premature leaf senescence. Marker genes associated with senescence, such as autophagy-related protein 8 (ATG8) and superoxide dismutase genes, showed altered expression patterns, suggesting that infected leaves age and deteriorate more quickly than healthy ones. Additionally, metabolic changes, including increased lignin production and secondary metabolite accumulation, suggest that the plant is responding to stress by strengthening its cell walls and modifying its defense mechanisms (Tan *et al*. 2025). These findings indicate that phytoplasma infection triggers premature senescence and structural changes as stress responses, leading to leaf size reduction and bushy growth (witches'-broom).

Integrating omics-based and targeted molecular studies enhances understanding of phytoplasma-host interactions, guiding strategies for disease resistance and management in sweet cherry and other crops. Future research should focus on identifying resistance genes, effector targets, and metabolic pathways to develop phytoplasma-resistant crops and targeted disease control interventions.

PHYTOPLASMA TAXONOMY: NOMENCLATURE, CLASSIFICATION, AND IDENTIFICATION

Historical background and challenges in phytoplasma classification

Phytoplasmas were historically misclassified as viruses due to their uncultivable nature. In 1967, researchers identified them as bacteria which were initially referred to as mycoplasma-like organisms (MLOs) due to their resemblance to mycoplasmas, which infect animals (Doi *et al*. 1967). In 1993, the International Committee on Systematic Bacteriology (ICSB) formally designated them as phytoplasmas, emphasizing their role as plant pathogens (International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of Mollicutes 1993).

One of the biggest challenges in phytoplasma research is their lack of cultivability, making identification, classification, and characterization particularly difficult. Early classification attempts were based on symptomatology, host range, insect vector specificity, and serological markers. However, these approaches were inconsistent and unreliable due to the variability in symptom expression and host-pathogen interactions. The situation changed in the 1990s with the introduction of molecular and culture-independent genotypic approaches, which revolutionized bacterial systematics, including the study of both culturable and non-culturable bacteria like phytoplasmas (Wei & Zhao 2022).

Phytoplasma nomenclature

Since phytoplasmas cannot be grown *in vitro*, they are currently accommodated under the genus '*Candidatus* Phytoplasma' (Murray & Schleifer 1994). The first formally recognized '*Candidatus* Phytoplasma' species was '*Ca*. Phytoplasma aurantifolia' in 1995, associated with lime witches'-broom disease (Zreik *et al*. 1995).

For decades, phytoplasma naming was primarily based on 16S rRNA gene sequencing, with a demarcation threshold of 97.5% sequence identity to define species (IRPCM Phytoplasma/Spiroplasma Working Team- Phytoplasma Taxonomy Group 2004). However, as sequencing technologies advanced, whole-genome sequencing (WGS) emerged as a more precise classification method. In 2022, the following new guidelines were introduced for phytoplasma taxonomy: (1) Increase the 16S rRNA sequence identity threshold from 97.5% to 98.65% for species-level differentiation, (2) Introduce whole-genome average nucleotide identity (ANI) with a threshold of 95–96% as a key classification criterion, (3) Incorporate multi-locus sequence analysis (MLSA), using conserved housekeeping genes such as *groEL*, *tuf*, *secA*, and *secY* to distinguish closely related species (Bertaccini *et al*. 2022; Wei & Zhao 2022).

Database-guided phytoplasma classification

Phytoplasmas are primarily classified using the 16Sr group/subgroup system, which is based on restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA gene (Lee *et al*. 1995). This method, developed in the 1990s, allows researchers to group phytoplasma strains into well-defined categories based on genetic similarity. An online tool called *i*PhyClassifier has been developed to facilitate this classification, allowing scientists to automate the process of assigning new phytoplasma strains to existing groups (Fig. 5; Wei *et al*. 2007, 2008b; Zhao *et al*. 2009). *i*PhyClassifier is a user-friendly online tool that allows researchers to (1) assign species under the '*Candidatus* Phytoplasma' system, (2) classify phytoplasmas into 16Sr groups and subgroups, (3) calculate RFLP similarity coefficients, (4) generate virtual gel images for comparative analysis, and (5) compare RFLP patterns with reference strains.

By simply inputting a phytoplasma 16S rRNA gene sequence, users can obtain automated classification results, making phytoplasma taxonomy more efficient and standardized. Using *i*PhyClassifier, researchers have identified emerging and reemerging phytoplasma diseases, improving disease surveillance and classification accuracy.

Currently, phytoplasmas are divided into 37 groups and more than 150 subgroups. Each group typically corresponds to a specific disease and geographical distribution (Wei & Zhao 2022). For example, the 16SrI (Aster Yellows Group) includes phytoplasmas that infect a wide variety of crops, including wheat, lettuce, and tomatoes. However, classification remains challenging, as some phytoplasma strains share high genetic similarity but differ significantly in ecological behavior, host range, and insect vector specificity. The integration of whole-genome sequencing into phytoplasma classification is expected to resolve many of these ambiguities and improve species differentiation.

Phytoplasma identification and diagnostic methods

Since phytoplasmas cannot be cultured, their identification relies entirely on molecular diagnostic techniques. Among the most widely used methods, PCR-based detection remains the standard approach for detecting phytoplasma DNA in infected plant tissues. Conventional PCR is commonly employed to amplify phytoplasma DNA, providing a reliable means of detection (Lee *et al*. 1993). However, when phytoplasma concentrations are low, nested PCR is often used for its enhanced sensitivity,

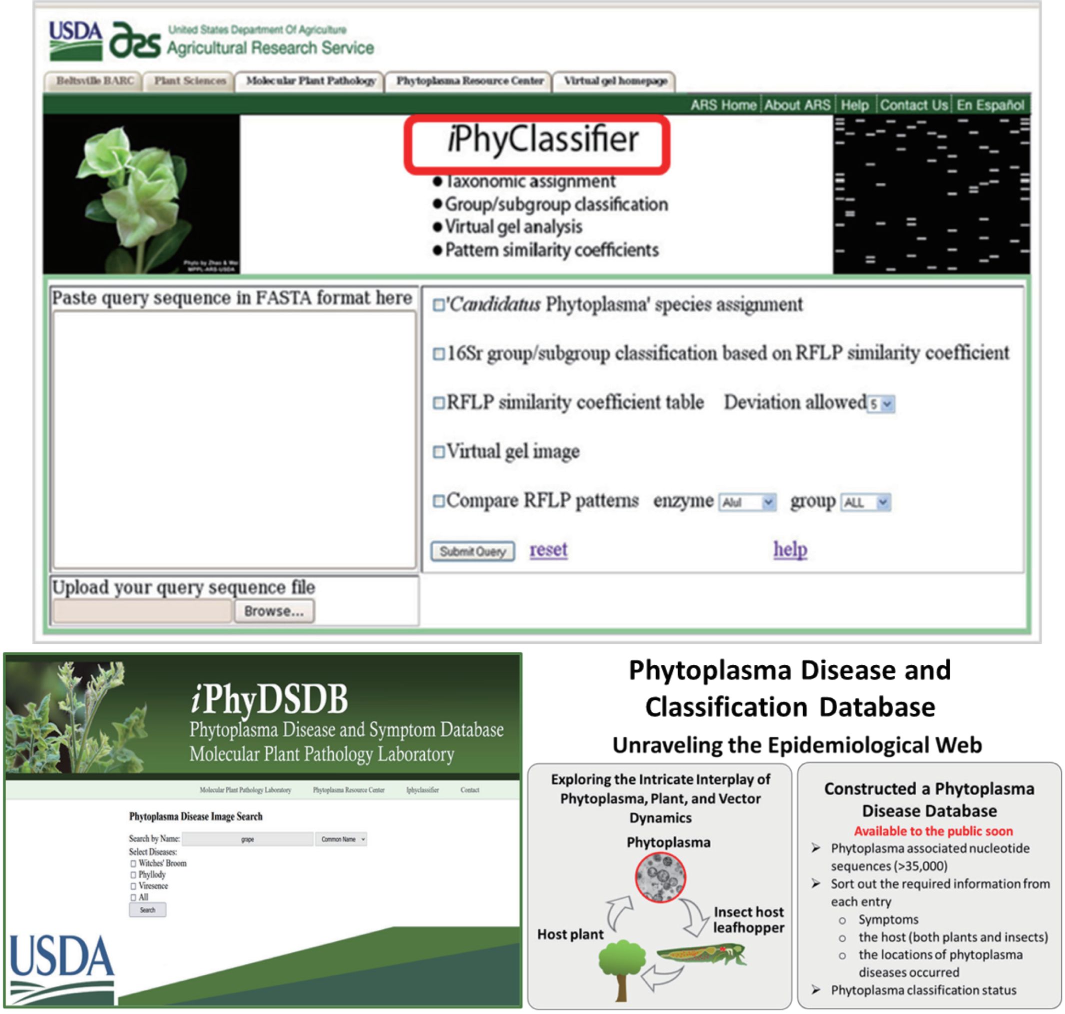

Fig. 5. Database-guided classification, identification, and epidemiological prediction of emerging and reemerging phytoplasma diseases.

enabling more accurate detection in samples with minimal pathogen presence (Gundersen & Lee 1996). More advanced methods, such as PCR, nested PCR, real-time PCR (qPCR, Christensen *et al*. 2004; Wei *et al*. 2004) and droplet digital PCR (ddPCR, Mehle *et al*. 2014), enable quantitative detection, offering deeper insights into disease progression and pathogen load in infected plants.

In addition to PCR-based methods, recent advancements in field-based diagnostics have significantly improved the speed and accuracy of phytoplasma detection. Loop-mediated isothermal amplification (LAMP) has emerged as a rapid and cost-effective technique that does not require sophisticated laboratory equipment, making it suitable for on-site diagnostics (Dickinson 2015). Another cutting-edge technology, CRISPR-based detection, leverages CRISPR-Cas systems to identify phytoplasma DNA with high specificity and accuracy (Wheatley *et al*. 2022). This approach holds great promise for real-time, field-deployable diagnostics, allowing for quicker disease management and intervention in agricultural settings.

AI AND DATA SCIENCE IN PHYTOPLASMA RESEARCH: ENHANCING DISEASE MONITORING

AI-based detection of phytoplasma diseases

Traditional diagnostic methods, such as polymerase chain reaction (PCR), and quantitative PCR, are time-consuming, labor-intensive, and require specialized laboratory equipment, making them impractical for most farmers, particularly in resource-limited settings. As a result, there is a growing need for rapid, accessible, and cost-effective diagnostic solutions. Our group explores an AI-driven system utilizing Convolutional Neural Networks (CNNs) to analyze plant images for early and efficient disease detection, enabling timely intervention for improved crop management (Wei *et al*. 2024b; Wei *et al*. 2025).

A case study was conducted on tomato plants infected by PPT phytoplasma. A comprehensive dataset was compiled, consisting of thousands of images capturing both healthy and infected plants. To ensure the reliability of the model, 20% of the dataset was reserved for testing and validation. Five CNN architectures were trained using transfer learning: four pre-trained models (VGG-16, Google Inception v3, NASNet, and DenseNet201) and one custom model specifically designed for phytoplasma detection. The pre-trained models leveraged weights from ImageNet, which provided a strong foundation for feature extraction and image classification. In contrast, the custom model was developed from scratch to capture unique morphological changes associated with phytoplasma infections. To enhance model robustness and reduce overfitting, TensorFlow was used to implement data augmentation techniques, including image rotation, flipping, and contrast adjustments. These preprocessing techniques helped simulate real-world variations in plant appearances, ensuring that the model generalizes well to diverse conditions (Wei *et al*. 2025).

The pre-trained models exhibited strong performance due to their extensive prior training on large-scale image datasets, allowing them to effectively distinguish between healthy and diseased plants. Among the pre-trained models, DenseNet201 and NASNet demonstrated the highest classification accuracy, demonstrating their ability to recognize subtle visual patterns indicative of phytoplasma infections. The custom model, while requiring more data and training time, provided valuable insights into phytoplasma-specific markers and offered a more tailored approach to disease identification (Wei *et al*. 2025). To enhance diagnostic accuracy and overall reliability, ensemble learning techniques were implemented. By combining the predictions of multiple models using majority and weighted voting strategies, the system mitigated the limitations of individual models and significantly improved classification performance. The ensemble approach effectively reduced false positives and false negatives, ensuring more consistent and dependable results across varied image inputs (Wei *et al*. 2025). Future research should focus on expanding the dataset to include a wider range of plant species and different phytoplasma diseases.

Phytoplasma Disease and Symptom Database (*i*PhyDSDB)

In the meantime, to support farmers and growers in recognizing diseases in their crops, we developed an image and symptom database known as the Phytoplasma Disease and Symptom Database (*i*PhyDSDB, Fig. 5; Wei *et al*. 2024a). This database contains a comprehensive collection of plant symptom images and associated data. It serves as a critical backup tool, allowing users to compare symptoms observed in their crops with those in the database, helping them to identify potential infections early on. By offering this interim solution, we aim to empower farmers and gain recognition while the AI-based system undergoes further development. Ultimately, the combination of the AI

model and this robust database will provide an integrated, scalable approach to managing phytoplasma diseases and safeguarding crop production.

Phytoplasma Disease and Classification Database

A Phytoplasma Disease and Classification Database is under construction (unpublished). This is a searchable database that links each isolated case and provides the users with a comprehensive view, making it possible to quickly compare various phytoplasma diseases from symptoms, host, and classification status. The Phytoplasma Disease and Classification Database is very helpful for early warning of the occurrence of phytoplasma diseases and provides the basis for the formulation of effective prevention and control measures.

In recent years, the introduction of drone technology has transformed phytoplasma research by enabling efficient sample collection from previously inaccessible regions, improving the identification of new phytoplasma strains and enhancing disease epidemiology studies. As plant and insect sampling increases, so will discoveries of novel phytoplasmas, further expanding our understanding of their distribution and impact. Big data analytics has already demonstrated its potential in tools such as *i*PhyClassifier, *i*PhyDSDB, and the Phytoplasma Disease and Classification Database, streamlining taxonomy and disease monitoring. Moving forward, the integration of big data, knowledge networks, and precision agriculture technologies will enhance research depth and improve disease management strategies.

CONCLUSIONS

The combination of top-down multi-omics and bottom-up molecular analyses has advanced phytoplasma research, revealing key molecular mechanisms of pathogenesis and symptom development. Innovations in whole-genome sequencing, molecular detection tools, AI-driven surveillance, and big data analytics continue to refine taxonomy, diagnostics, and disease management. Sustained interdisciplinary collaboration will be crucial for developing effective and sustainable strategies to mitigate phytoplasma-related agricultural loss.

ACKNOWLEDGEMENTS

Sincere appreciation is extended to all team members and collaborators for their dedication, hard work, and contributions. The work was primarily supported by the US Department of Agriculture, Agricultural Research Service (Project number 8042-22000-320-00D).

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由上而下與由下而上的植物菌質體研究：致病機制、分類學及診斷技術之進展

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摘要

Wei, W. 2025. Top-down and bottom-up approaches in phytoplasma research: advancing pathogenesis, taxonomy, and diagnostics. J. Taiwan Agric. Res. 74(4):359–376.

植物菌質體為微小且無細胞壁的細菌，可導致毀滅性的植物病害，造成農業重大經濟損失。本文探討植物菌質體的最新研究進展，重點在於其致病機制、分類學以及透過由上而下與由下而上的診斷方法。由上而下的多組學研究 (multi-omics studies)，對於植物菌質體所引起的干擾 (尤其是在糖代謝與賀爾蒙傳導方面) 提供系統層次的理解，揭示其對植物生理之廣泛影響。除此，由下而上的研究策略則剖析了分子相互作用，闡明植物菌質體如何干擾分生組織的歷程、如何調節植物生長模式，以及如何改變植物結構並誘發可作為區辨特徵的危害徵狀。分類學 (taxonomy) 與特徵歸類的分類法 (classification) 的進步改善了物種分化，整合了 16S rRNA 定序、多位點序列分型 (multilocus sequence typing; MLST) 及全基因組定序 (whole-genome sequencing; WGS)，並透過資料庫指導工具提高了分類準確性。基於 clustered regularly interspaced short palindromic repeats-based (CRISPR) 的檢測等尖端診斷技術的發展，顯著提高了植物菌質體鑑定與監測的敏感度、特異性及效率。此外，大數據分析與人工智慧驅動模型的整合，開創了基於影像的症狀識別技術，為疾病監測與監控提供支援。本文整合了關鍵研究成果與技術進步，展示了整合由上而下的系統生物學 (systems biology) 與由下而上的分子分析，如何創新推動植物菌質體的檢測、分類以及永續的病害管理策略。

關鍵詞：分生組織命運偏離、*i*phy 分類系統、人工智慧、組學、花期轉換。

投稿日期：2025 年 6 月 16 日；接受日期：2025 年 8 月 13 日。

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台灣農業研究 (J. Taiwan Agric. Res.) 74(4):377–397 (2025)
https://doi.org/10.6156/JTAR.202512_74(4).0002

Feature Article

Bacteria in Insect Pest Management in Agriculture: The Past, Present, and Future

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Abstract

Burbank, L. P. and A. Guerrero. 2025. Bacteria in insect pest management in agriculture: The past, present, and future. J. Taiwan Agric. Res. 74(4):377–397.

Insect pests have plagued agriculture throughout history, and management of crop pests requires continuous innovation. Bacteria and other microbes are important tools for insect pest control through pathogenic activity, production of toxic secondary metabolites, and promotion of plant defenses. Additionally, bacteria present in agricultural environments often possess the ability to metabolize agricultural chemicals and can therefore be used to mitigate environmental impacts of agriculture. Only a small percentage of bacteria from plant and insect sources have been studied however, while there is a wealth of microbial diversity that is still unused. As the need for agricultural productivity increases and climate change shifts patterns of pest and disease, it is important to explore a wider range of bacterial species with potential for biological pest control and bioremediation applications. This review outlines some of the history of bacterial biological control, use of bacteria-derived agrochemicals, bacteria-mediated insecticide resistance, and current understanding of bacteria-insect-plant interactions. Suggestions are also provided for further research in this area to broaden the range of sustainable pest control options.

Key words: Insecticides, Biological control, Agriculture, Beneficial bacteria, Pest management.

BACKGROUND: BACTERIA AS PART OF INSECT PEST MANAGEMENT

Plant pests and pathogens have a significant impact on food security (Flood 2010). Although it is challenging to estimate across diverse crops and agricultural systems, insect pests in general and invasive insects in particular cost billions of dollars in losses and management investments globally (Bradshaw *et al*. 2016). For centuries, crop production has relied on the use of insecticidal compounds of various types, the first ones based on metals and elements or botanical extracts (Oberemok *et al*. 2015). In the early 1900s, discovery of insecticidal activity from bacteria and bacterial products led to a series of microbial-based insect pest management strategies that continues today (Starnes *et al*. 1993; Federici 2005; Wend *et al*. 2024). With increasing use of synthetic chemical insecticides on crop plants in the last 70 years, insecticide resistance and environmental damage emerged as concerning issues (Forgash 1984; Geiger *et al*. 2010; Brühl & Zaller 2019; Sparks *et al*. 2021b). Advancements in biotechnology have kept bacteria-based insec-

Received: June 16, 2025; Accepted: September 25, 2025.
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ticides relevant in pest management throughout this trajectory in the form of modified natural products (Kirst 2010), biological controls (Faria *et al*. 2023), and bacterial genes expressed in plants (Gassmann & Reisig 2023). Although not exempt from concerns over resistance and environmental impact (Tabashnik *et al*. 2013), the diverse bacterial species that interact with insects and insecticidal compounds remain an abundant resource for new and improved pest management strategies. In addition to direct uses as biological control, bacteria interface with agricultural systems in many ways including through soil and plant microbial communities, contamination of food products, and remediation of the environmental impacts of agriculture. Understanding the role and potential of bacteria in all these areas will contribute to development of more sustainable and resilient crop management practices. The goal of this review is to highlight some of the important roles of bacteria in insect pest management systems of the past and present, and to discuss methods and future directions for research in this area.

INSECTICIDES DERIVED FROM BACTERIA

Many potent insecticidal compounds commonly used in agricultural pest management are natural products produced by bacteria. These compounds can be produced and extracted from the bacteria themselves, produced transgenically in plants, or modified and chemically synthesized. Although these are not the only bacterial-derived insecticides, Bt, abamectin, and spinosyns are discussed here as examples of widely used commercial insecticide products of bacterial origin.

Long history of Bt toxin

One of the most widely used bacterial insecticides is a group of toxins produced by the diverse bacterial species *Bacillus thuringiensis* (Bt). *B. thuringiensis* includes several subspecies of gram-positive spore-forming bacteria that can be found in the environment (Nester *et al*. 2002). Crystals produced by the bacteria during the spore formation process are highly toxic to certain insects if ingested. Additional toxins can also be produced as the bacterial spores germinate and reproduce within insects (Li *et al*. 2024). Insecticidal properties of Bt were first identified over 100 years ago, and Bt products have been used in agriculture since the 1930s (Lambert & Peferoen 1992). One of the advantages of Bt toxins is their specificity for certain insects and limited toxicity to mammals (de Maagd *et al*. 2001). This specificity allows Bt to be used in integrated management programs and in combination with biological controls. Although impacts on non-target organisms are generally limited, there may still be some risks for sub-lethal or secondary impacts on specific non-target insects such as parasitoids (Yu *et al*. 2011). Many of the insect pests most effectively targeted belong to the lepidopteran, dipteran, and coleopteran orders, although uses against other insect orders such as hemipterans, and some nematode species are still being explored (Federici *et al*. 1990; Baum *et al*. 2012; Domínguez-Arrizabalaga *et al*. 2020; Ramalakshmi *et al*. 2020; Pinheiro & Valicente 2021).

In addition to Bt sprays and bacterial spore formulations, the genes which produce the toxic crystal proteins were introduced transgenically into several different crop plants, successfully conveying resistance to insect damage (Dively *et al*. 2018). Plant modification with Bt improved efficacy since Bt sprays and external products break down quickly in the environment. In the United States, adoption of Bt modified crops started in the late 1990s, and is currently at 80–90% of planted acres for some crops such as cotton and corn (USDA-ERS 2024). There is also significant commercial use of Bt crops in South America (Brookes 2018; Páez Jerez *et al*. 2023) and Asia (Kathage & Qaim 2012; Zhang & Dong 2024). Although field crops such as corn, soybean, and cotton are the most widely used Bt plants, Bt modified vegetables such as eggplant are also produced in some areas (Ahmed *et al*. 2024). However, key insect pests rapidly

developed resistance to the first generation of Bt crops, prompting development of a second generation of the technology that includes multiple toxin genes instead of a single toxin. Combined with appropriate management practices such as use of non-Bt refuge plants alongside the Bt crop, resistance development can be slowed but not eliminated completely (Tabashnik *et al.* 2013).

Despite the long history of Bt in agricultural pest management, only a few bacterial strains out of the diverse *B. thuringiensis* species have been used and developed into pest control products. New strains continue to be characterized, including many that target new pest species. However, there is potentially many significant and diverse Bt products have not been explored yet for agricultural use. *B. thuringiensis* and related species can be found in soil, associated with insects, growing on plant surfaces, and as an endophyte in wild and cultivated plants (Espinoza-Vergara *et al.* 2023). There is also evidence for beneficial traits beyond insecticidal capacity including plant growth promotion and pathogen suppression for some members of the *B. thuringiensis* species (Cherif-Silini *et al.* 2016).

Bacterial chemistry solutions- avermectins and spinosyns

Many bacterial species, particularly those found in soils, produce a wide range of secondary metabolites with antimicrobial and insecticidal properties. Several important chemical insecticides used in agriculture have emerged from this niche. The genus *Streptomyces* is well known for its secondary metabolite production and is the source of many antibacterial and antifungal compounds (Kemung *et al.* 2018). The species *S. avermitilis* also produces a group of chemicals called avermectins which were originally identified as nematicides against animal parasites (Burg *et al.* 1979). Avermectins and similar natural products from *S. avermitilis* have a wide range of insecticidal activities and are effective as a foliar spray against pest insects of plants, as well as mites and nematodes (Putter *et al.* 1981; Bull 1986). These compounds belong to the family of macrocyclic lactones, which have relatively safe profile for humans and vertebrate animals (Bai & Ogbourne 2016). Ivermectin, of the same chemical family, is even used extensively as a pharmaceutical in veterinary and human medicine (Õmura & Crump 2004). Synthetic derivatives have also been produced from avermectin compounds with improved properties such as water solubility and thermal stability. The derivative salt, emamectin benzoate, is one of these synthetic derivatives with improved efficacy against lepidopteran pests (Ishaaya *et al.* 2002).

Another macrocyclic lactone produced by bacteria and extensively used in pest control is spinosad. Products made from spinosad have been sold in several commercial formulations since the 1990s and were originally identified from the actinomycete bacterial species *Saccharopolyspora spinosa* (Mertz & Yao 1990; Kirst 2010). Spinosad, which contains major and minor fermentation products spinosyn A and spinosyn D, is extremely potent against a wide range of insects including key agricultural pests such as thrips, fruit flies, European corn borer, fall armyworm, and leafminers (Biondi *et al.* 2012). Additional spinosyn variants, the butenyl-spinosyns, were identified from related bacterial species *Saccharopolyspora pogona* (Lewer *et al.* 2009). Generally considered safe for non-target species, the spinosyns are registered for use on organic crops in multiple countries (USDA-AMS 2022; Micheloni *et al.* 2023). These original bacterial products also became the basis for synthetic modification to create improved chemical variants. Use of artificial neural networks and quantitative structure activity relationships led to development of spinetoram, a compound with improved and longer lasting insecticidal activity (Dripps *et al.* 2008; Sparks *et al.* 2021a). Spinosad and spinetoram have been used in commercial agriculture since 1997 and 2007 respectively, generally with great success. However, overuse can lead to generation of resistant pest populations, and this has been observed for diamondback moth

in *Brassica* crops, olive fruit fly, and beet armyworm among others (Moulton *et al*. 2000; Zhao *et al*. 2002; Kakani *et al*. 2010). More recent evidence also suggests that although acute toxicity to pollinators and beneficial insects is limited, sublethal exposure can have negative impacts (Biondi *et al*. 2012; Martelli *et al*. 2022).

Avermectins and spinosad are just some of the bacterial secondary metabolites with potential for pest control use. As with other chemical control strategies, development of resistance in insect populations does occur with these natural products and it is important to continue to investigate novel solutions. Like antibiotic discovery for human medicine, analyzing bacteria from extreme and unusual environments could be a path to identification of new bacterial products for pest control (Quinn & Dyson 2024).

BACTERIA FOR BIOLOGICAL CONTROL

Aside from purifying, modifying, and synthesizing bacteria-derived metabolites, in many cases it can be very successful to use the bacteria themselves to infect or inhibit insect pests. One advantage of using the biological organism is that it can replicate on its own and potentially persist in the environment for longer periods of time. On the other hand, living organisms must remain viable to be effective, and are strongly influenced by environmental conditions and selective pressures.

Insect-pathogenic bacteria

Like other animals, insects are threatened by a wide range of pathogens including fungi, viruses, and bacteria. Entomopathogenic bacteria can infect insects by ingestion and cause systemic infection or disruption of feeding that leads to insect starvation (Ruiu 2015). Some of these are free-living opportunistic bacteria that colonize insects causing lethal infections (Flury *et al*. 2016). *Pseudomonas fluorescens* strains Pf-5 and CHA0 are two such soil-dwelling bacteria that can colonize and kill the larvae of *Galleria mellonella* and *Manduca sexta* (Péchy-Tarr *et al*. 2008). Other related species such as *Ps. chlororaphis* and *Ps. protegens* are also pathogenic to lepidopteran larvae (Flury *et al*. 2016). The genus *Bacillus* includes many bacteria with biological control potential against insects. For example, longevity of the sorghum pest Southern green stink bug, *Nezara viridula*, was reduced after feeding on *B. velezensis* (Esquivel *et al*. 2022). In addition to free-living soil bacteria, plant endophytes can also have insect-pathogenic traits. Although more endophytic fungi were studied extensively, reduced pest damage was observed in rice plants colonized by an entomopathogenic strain of the bacterial species *Serratia marcescens* (Niu *et al*. 2022). However, the effectiveness of entomopathogenic bacteria for pest control is complex in many cases as it depends on insect life stage, as well as whether the bacteria are used in combination with other compatible pest control products (El Fakhouri *et al*. 2023). Pathogenic mechanisms of bacteria infecting insects often include production of enzymes such as proteinases and chitinases which facilitate invasion of insect tissues. Production of these enzymes can be used as a screening tool to identify entomopathogenic bacteria with biological control potential (Dai *et al*. 2024).

In addition to the generalist species living in soil and plant environments, other entomopathogenic bacteria have highly specialized lifestyles, such as those associated with predatory nematodes. *Photorhabdus luminescens* is one such bacterial species which forms a symbiotic relationship with nematodes of the genus *Heterorhabditis* (Dunphy & Webster 1988). When the nematodes invade an insect larva, *Ph. luminescens* is injected into the insect hemocoel where it causes infection and death of the insect (Rajagopal & Bhatnagar 2002). This infection process occurs through secretion of bacterial toxins and virulence factors that facilitate decomposition of the larval body so that the bacteria can acquire nutrients (Rodou *et al*. 2010). Some of these toxins produced by *Ph.*

luminescens are also toxic to adult insects via ingestion and have been explored for targeting agricultural pests such as white flies (*Bemisia tabaci*) and Colorado potato beetle (*Leptinotarsa decemlineata*) (Blackburn *et al*. 2005).

Although several bacterial species have been identified for biological control of various insect pests, few of them enter the market as commercial products. In many jurisdictions, microbial pesticides such as entomopathogens and biological control products are subject to specific and rigorous regulatory processes like chemical pesticides (Wend *et al*. 2024). In other areas, farmers may take it on themselves to produce microbial cultures for pest control directly on their farms (Faria *et al*. 2023). Quality of on-farm biopesticide production varies depending on expertise and equipment, however. In most places, widespread adoption of bacterial insect control products requires commercialization and production at large scale. This can be more challenging for microbial products than for chemical products since the organisms must remain viable under storage and transport conditions, and efficacy is highly reliant on appropriate environmental conditions (Teixidó *et al*. 2022; Koul 2023).

Protective effects of phytobiomes-bacterial community interactions

Although more commonly associated with plant pathogen suppression, beneficial components of the phytobiome, or microbial communities associated with plants, can impact insect feeding and therefore pest damage. The phytobiome encompasses microbes living on plant surfaces both above and below ground, as well as endophytes within the plant. Bacteria from all these niches can impact insect pests in different ways, but often involve plant defense responses. Disease-suppressive soils containing strains of *Ps*. *protegens*, were able to protect wheat plants from the cereal leaf beetle *Oulema melanopus* (Almario *et al*. 2014; Harmsen *et al*. 2024). This effect was attributed to plant defense priming and possibly reduced insect fitness due to disruption of the insects' beneficial symbiotic bacteria (Harmsen *et al*. 2024). Likewise, *Arabidopsis* plants grown in soil with different microbiome compositions were found to have changes in chemical metabolites which correlated with reduced feeding of cabbage looper (*Trichopulsia ni*) larvae (Badri *et al*. 2013). In some cases, the impact of rhizosphere microbiome composition can be greater than plant genetic variability in determining resistance to herbivorous insects (Hubbard *et al*. 2019). What distinguishes microbiome-mediated resistance to insect pests from direct pathogenesis by entomopathogenic bacteria, is that it typically occurs indirectly through physiological and metabolic changes in the plant. In addition to phytohormone signaling and production of secondary metabolites, presence of certain bacteria can trigger structural changes such as reinforcement of cell walls that also deters insect feeding (Underwood 2012). Because of the systemic nature of plant-microbe and plant-insect interactions, insect pest feeding and damage can be influenced by microbial interactions that occur in the soil as well as above-ground plant parts (Reverchon & Méndez-Bravo 2021). In some cases, soil microbial communities can impact the plant nutritional value and palatability to insects through influence on nutrient uptake (Poveda *et al*. 2005). Nitrogen-fixing bacteria such as *Rhizobium* are essential for optimal plant growth and performance of many plant species in the legume family. Nitrogen content in leaf tissues is also important for production of alkaloid compounds which are components of the plant defense response to herbivore feeding (Irmer *et al*. 2015; Liu *et al*. 2020). Cultivation of lima bean plants with rhizobia increased production potential of these secondary metabolites and in turn reduced feeding of beetles (Thamer *et al*. 2011). Soil treatment with plant growth-promoting *Bacillus* sp. also reduced infestation of cabbage aphid (*Brevicoryne brassicae*) in *Brassica oleracea* plants (Gadhave *et al*. 2016). Although less well-studied for plant health promotion than rhizosphere colonizing bacteria, there are also some species of phyllosphere bacteria that are correlated with reduced

insect damage (Humphrey *et al*. 2014). Many of these beneficial bacterial benefits are effective in a field scenario, and not just under strictly controlled plant growth conditions (Heinen *et al*. 2018).

BACTERIAL DEGRADATION OF INSECTICIDE COMPOUNDS

Two of the main challenges of insecticide use in agriculture are development of resistant insects and lasting environmental impacts of agrochemicals. Diverse bacterial species present in the environment can metabolize many natural and synthetic agrochemicals. This ability can be exploited for remediation of environmental contaminants in both agricultural and industrial processes. However, capability of bacteria to digest insecticidal compounds can also contribute to insect tolerance to these chemicals, in some specific cases that have been studied.

Insecticide tolerance facilitated by symbiotic bacteria

Like other animals, insects harbor a range of microorganisms within and on their bodies (Douglas 2015). Some of these are obligate intracellular symbionts, but many others are acquired from the environment and reside in open niches such as the outer cuticle and digestive system. Bacterial species found in the gut of insects have been implicated in detoxification of plant compounds, and more recently research has identified bacteria that also detoxify pesticides used in agriculture (Table 1) (Adams *et al*. 2013; Douglas 2015; Wang *et al*. 2022). The dynamics and mechanisms behind these interactions of insects, bacteria, and pesticides are still largely under investigation, but there is enough existing evidence from specific insect-bacteria relationships to consider this possibility when insecticide tolerance is observed in agricultural pests.

Bacteria capable of metabolizing insecticidal chemicals are found in plant and soil environments and can colonize insects in a stable manner throughout the lifecycle. In multiple different pest species, symbiotic associations with these bacteria have been shown to convey resistance to specific insecticides. Bean bug (*Riptortus pedestris*) acquires symbiotic *Burkholderia* that can degrade the organophosphate insecticide fenitrothion (Kikuchi *et al*. 2012). These bacteria can accumulate in soil where fenitrothion is used extensively in agriculture and be naturally acquired by pest insects. Similar dynamics were also observed in the rice stinkbug (*Cletus punctiger*) when studied under laboratory conditions (Ishigami *et al*. 2021). In the brown planthopper (*Nilaparvata lugens*), different variants of the symbiont bacterium *Arsenophonus* provided different levels of resistance of the insects to imidacloprid (Pang *et al*. 2018). In this case, some specific *Arsenophonus* strains decreased resistance in the insects in laboratory studies, suggesting that there may be potential to increase pest control efficacy through dissemination of specific bacteria in the environment (Pang *et al*. 2018). Diamond-back moth (*Plutella xylostella*) harbors several different bacterial species which affect tolerances to the commonly used insecticide chlorpyriphos (Xia *et al*. 2018). Particularly some strains of *Enterococcus* were able to increase resistance, in this case through effects on the insect immune system and production of antimicrobial peptides in addition to direct degradation (Xia *et al*. 2018). Likewise, in *N. lugens*, *Wolbachia* symbionts increased insect stress response to sublethal doses of imidacloprid and may help the insects adapt to this chemical exposure (Cai *et al*. 2021). Although specific details of these interactions have mostly been characterized in laboratory experiments, in some instances there is evidence of bacterial populations with insecticide degradation ability acquired through selection of an insect population in agricultural settings. For example, selection in the field of *Spodoptera frugiperda* larvae led to more diverse bacterial populations with increased capabil-

Table 1. Major insecticide classes for which bacterial degradation has been identified.

Insecticide class[z]	Bacteria-mediated resistance in insects	Source of bacteria	Bacterial genera	Reference
Carbamate (carbaryl, isoprocarb, fenobucarb, carbofuran)	Unknown	Wastewater, landfill, soil	*Pseudomonas*, *Cupriavidus*, *Paracoccus*, *Rhizhobium*, *Sphingomonas*, *Achromobacter*, *Novosphingobium*	Tomasek & Karns (1989); Feng *et al*. (1997); Hashimoto *et al*. (2002); Yan *et al*. (2007); Peng *et al*. (2008); Trivedi *et al*. (2016); Gupta *et al*. (2019)
Neonicotinoid (Acetamiprid, imidacloprid, thiamethoxam)	Yes	Soil, water, plant, wastewater, insect	*Pigmentiphaga*, *Acinetobacter*, *Sphingomonas*, *Stenotrophomonas*, *Ensifer*, *Bacillus*, *Pseudomonas*, *Mycobacterium*, *Leifsonia*, *Klebsiella*, *Hymenobacter*, *Enterococcus*	Anhalt *et al*. (2007); Pandey *et al*. (2009); Wang *et al*. (2011); Tang *et al*. (2012); Phugare *et al*. (2013); Wang *et al*. (2013); Zhou *et al*. (2013); Sharma *et al*. (2014); Kandil *et al*. (2015); Rana *et al*. (2015); Guo *et al*. (2020); Sivakumar *et al*. (2022); Lv *et al*. (2023)
Organochlorine (lindane, chlordane)	Unknown	Soil, water	*Streptomyces*, *Micromonospora*	Fuentes *et al*. (2010); Raimondo *et al*. (2020)
Organophosphate (dimethoate, fenitrothion, chlorpyrifos, Trichlorpho)	Yes	Insect	*Serratia*, *Burkholderia*, *Enterococcus*, *Citrobacter*, *Pseudomonas*	Boush & Matsumura (1967); Kikuchi *et al*. (2012); Cheng *et al*. (2017); Xia *et al*. (2018); Ishigami *et al*. (2021); Xia *et al*. (2023)
Pyrethrins and pyrethroids (Bifenthrin, Deltamethrin, Cypermethrin)	Yes	Soil, wastewater, insect	*Serratia*, *Acinetobacter*, *Brevibacillus*, *Sphingomonas*, *Sphingobium*, *Achromobacter*, *Pseudomonas*, *Bacillus*, *Streptomyces*, *Ochrobactrum*, *Paracoccus*, *Enterococcus*, *Lactococcus*	Maloney *et al*. (1988); Guo *et al*. (2009); Lin *et al*. (2011); Zhai *et al*. (2012); Cycoń *et al*. (2014); Akbar *et al*. (2015); Pankaj *et al*. (2016); Tian *et al*. (2018); Yang *et al*. (2018); Gur Ozdal & Algur (2022); Zeng *et al*. (2024)
Macrocyclic lactone (abamectin, spinosad)	Unknown	Soil	*Stenotrophomonas*	Wang *et al*. (2015)

[z] Classified by chemical relatedness for regulatory purposes (US EPA 2013).

ities for degradation of multiple insecticides (Gomes *et al*. 2020).

Since insecticide-degrading bacteria can be selected for in the environment even in the absence of the pest, resistance can develop much more rapidly in an insect population that acquires these bacteria as symbionts compared with genetic resistance that may take extended periods of time to develop in an insect population (Kikuchi *et al*. 2012).

Bioremediation of agricultural environments

In some areas where pesticides have been used heavily, there is concern about toxicity in soil and water, and the impacts this may have on human health and biodiversity in the environment. Pesticide use overall is highest in the Americas and Asia, with insecticides being the second highest class of products used (Raffa & Chiampo 2021). Bioremediation involves use of biological organisms, often microbes, to degrade and metabolize these contaminants.

Bioremediation strategies include addition of beneficial organisms and stimulating growth of already present microorganisms with desirable metabolic properties (Gonçalves & da Silva Delabona 2022). There is evidence that soils can naturally accumulate higher degradation capacities after extended use of specific chemical inputs due to enrichment for bacterial species that degrade those chemicals (Jablonowski *et al*. 2013). Additionally, several decades of microbiology research has identified specific bacteria that can break down commonly used pesticides (Chaudhry & Ali 1988). Multiple bacterial genera have been identified for bioremediation capacity including *Pseudomonas*, *Burkholderia*, *Bacillus*, *Stenotrophomonas*, *Staphylococcus*, and *Streptomyces*. Many of these organisms were originally isolated from heavily contaminated environments (Fuentes *et al*. 2010). In some cases, genetic capability for insecticide degradation is carried on plasmids and can be horizontally transferred between different bacterial species rather than being associated with a single organism (Feng *et al*. 1997; Hayatsu *et al*. 2000; John *et al*. 2020). Many insecticide-degrading bacteria do so through production of specific enzymes which can also be utilized in a purified form for bioremediation efforts (Gangola *et al*. 2018; Jaffar *et al*. 2022). The benefit to using purified degradation enzymes rather than the original source bacteria, is that purified enzymes can be used for applications such as degrading pesticide residues on food (Fan *et al*. 2017).

One of the largest challenges to implementation of bacterial bioremediation is scaling up from small-scale experiments to field applications (Gonçalves & da Silva Delabona 2022). Like the challenges of bacteria in biological control, various factors in the field can impact results such as soil types and environmental conditions (Raimondo *et al*. 2020). Since many pesticide compounds are complex hydrocarbon molecules, full degradation often requires multiple different microbial species which can facilitate breakdown of various components and intermediate breakdown products (Ghazali *et al*. 2004; Yılmaz *et al*. 2022). In some cases such as neonicotinoids, complete breakdown is important as some of the initial breakdown intermediates are just as toxic as the original compound, and this may require facilitation by multiple microbial species (Dai *et al*. 2006; Ma *et al*. 2014; Pang *et al*. 2020). It is also likely that contaminated agricultural soils contain more than one chemical of concern, and that the environmental and microbial process needed for bioremediation will be complex.

METHODS FOR IDENTIFICATION OF BACTERIA WITH BIOLOGICAL CONTROL AND BIOREMEDIATION POTENTIAL

Although many beneficial bacteria have been identified for uses in insect pest control and agriculture, there is still significant bacterial diversity remaining untapped. General techniques

for isolation and identification of novel bacteria with useful traits are described here, many of which can be employed in low resource settings with only basic microbiology laboratory capacity (Fig. 1).

Direct culturing followed by screening

When the goal is to isolate a diverse range of bacterial species from a specific sample type such as insect or plant materials, direct culturing on rich and complex media can be used.

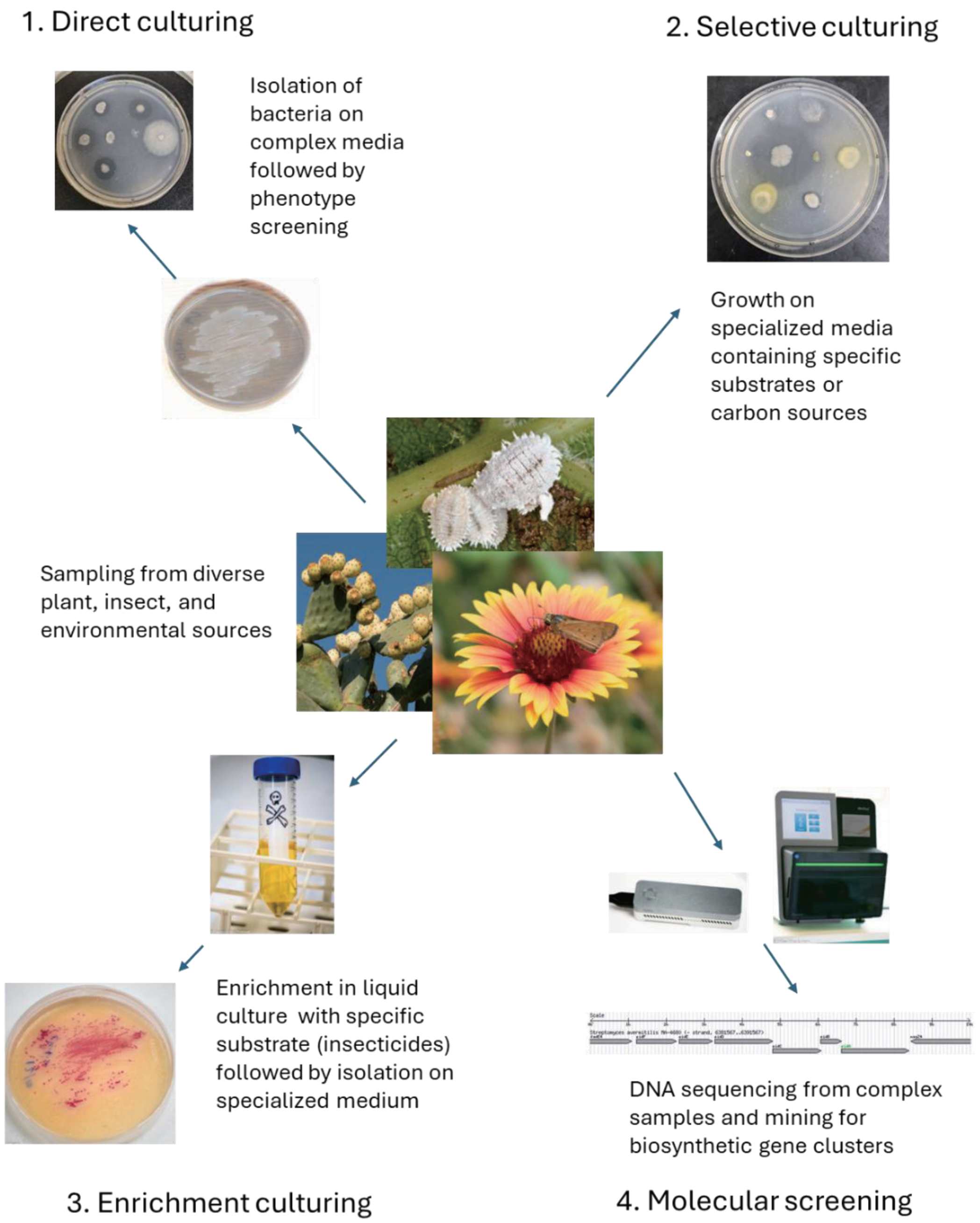

Fig. 1. Methods for identification of bacteria with biological control and bioremediation potential.

For direct culture experiments, samples of soil, plant, or insect materials are homogenized in sterile water or buffer and diluted onto a variety of complex culture media in Petri plates. Incorporating a range of media types, incubation temperatures, and atmospheric conditions, can yield more diverse bacterial isolates (Song *et al.* 2024). Once pure bacterial isolates have been obtained, these isolates can be screened for a variety of desirable traits such as production of antibiotics, chitinases, and carbohydrate-degrading enzymes (Pino-Hurtado *et al.* 2024; Zhang *et al.* 2024). However, direct culturing approaches will favor faster growing and more abundant organisms, and screening for desirable traits can be very time consuming. To address this challenge, various approaches to improving the diversity of organisms cultured from complex samples have been developed. Simple modifications to culture media can have significant impacts (Kato *et al.* 2018; Nishioka & Tamaki 2022), as can use of more complicated matrices instead of traditional Petri dish culture (Liu *et al.* 2021). Overall, the direct culturing approach typically needs to be tailored to the specific sample type, environment, and type of bacterial isolate desired.

Selective culturing

To selectively target bacterial isolates from a specific genus or with a specific growth trait or phenotype, growth media can be designed to target that trait or background. For example, sample pretreatments like desiccation, addition of selective antibiotics, and specific growth media can improve isolation of the genus *Streptomyces* (Oskay 2009; Antido & Climacosa 2022). For the genus *Pseudomonas*, there are also several semi-selective media formulations (Johnsen & Nielsen 1999), and other bacterial groups can be targeted if there are common chemical tolerances (Kini *et al.* 2019; Bonnet *et al.* 2020). Selecting for ability to utilize a specific carbon source can be done by plating dilutions of complex samples directly on minimal salt media including only that carbon source. This method has been used for isolation of chitinase producing organisms by using chitin as the sole carbon source (Saima *et al.* 2013), and for insecticide-degrading organisms by using a specific pesticide as the sole carbon source (Benimeli *et al.* 2007). The benefit to selective culturing is that bacteria of interest can be more quickly identified from complex samples. The drawback is that organisms may be excluded if they require supplemental nutrients, or if they are outliers for genus-specific growth requirements.

Enrichment culture

From complex samples such as soil or wastewater, enrichment culturing can increase the population of bacteria with the desired metabolic properties within the sample prior to isolating individual bacterial colonies. For this process, samples are typically mixed with minimal medium supplemented with the target chemical for degradation. Sequential culturing, sometimes with increasing concentrations of the target chemical, will select for species able to utilize the target as a carbon source (Akbar *et al.* 2015; Asamba *et al.* 2022). After several rounds of sequential culturing selection, samples are plated on minimal medium supplemented with the target chemical for isolation and identification of individual bacterial colonies. Benefits to this method are that rare species with the desired metabolic properties can be more easily identified, and minimal screening needs to be carried out afterwards if the selection process is robust. However, organisms that need a consortium of other bacteria or supplemental carbon or other nutrients to grow may be eliminated over the course of the selection process.

Molecular screening

Regardless of the growth medium and methods used, many bacterial species still cannot be cultured directly (Steen *et al.* 2019). Among the unculturable organisms, there is likely a significant number that have important biological traits that can be utilized indirectly. Metagenomic sequencing from a broader range

of samples and locations can identify new bacterial species and biosynthetic gene clusters (Medema *et al*. 2011). For many pesticide degradation pathways, the functional genes are known, and homologs can be identified from sequences. Novel degradation pathways can also be identified by cloning metagenomic DNA into libraries expressed in a model bacterial system and screening for activity (Ufarté *et al*. 2017), or by bioinformatic prediction (Jeffries *et al*. 2018; Malla *et al*. 2022). Although easily culturable bacteria can be more directly used as biological control organisms, the diversity of natural products and novel activity that is present in the non-culturable bacteria should not be overlooked.

FUTURE DIRECTIONS FOR RESEARCH

Around the world there has been increasing research investment and adoption of biological control and biopesticides in agriculture, and in mitigation of insecticide impacts (Gelernter 2007; van Lenteren & Cock 2020; Wyckhuys *et al*. 2020; Helepciuc & Todor 2022; Lopes *et al*. 2023; Pioneering proposals to restore Europe's nature by 2050). All this innovation is based on fundamental research identifying new biological control organisms, characterizing plant-microbe-insect interactions, and developing a detailed understanding of microbial products. There are still several areas where this research can be expanded, including identification of more diverse biological control organisms in general, focusing on locally adapted organisms for specific settings, and utilization of existing microbial collection resources. Over the last few decades, reduced costs and increased accessibility of microbial sequencing has significantly increased knowledge of bacterial species from different environments. Technological advancements in screening and identifying natural products have also facilitated discovery of new microbial compounds. However, samples from insect and plant sources are underrepresented in databases and collections compared with other sources such as human samples (Fig. 2). There is also significant bias in research investments towards known pest organisms, major crops, and high-resource countries (Manners & van Etten 2018; Bebber *et al*. 2019; Skaldina & Blande 2025). Increasing sampling diversity could uncover novel bacterial species with important biological traits. One of the most common reasons for biological control failure is influence of environmental conditions (Quesada-Moraga *et al*. 2024). Organisms that are not adapted to local climate may not survive well or provide adequate stability. A more specific focus on isolating organisms from the local area where they will be used has the potential to facilitate implementation and improve outcomes (Ningthoujam *et al*. 2009; Harman *et al*. 2010). It may also be necessary to re-assess organisms that were isolated many years ago, as the climate conditions where they were initially effective may have shifted. In addition to isolating new and more geographically targeted bacteria, there is a wealth of existing microbial resources in culture collections around the world. The World Federation of Culture Collections lists more than twenty formal collections (World Federation for Culture Collections, https://wfcc.info/sites/), and there are countless informal collections maintained by individual researchers globally. Organisms from these collections

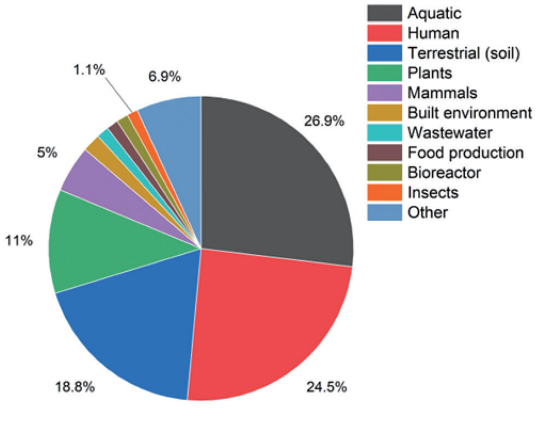

Fig. 2. Biosample type for all samples in Joint Genome Institute (JGI) Genomes Online Database (accessed December 2024).

can also be screened for relevant traits beyond the purpose they were collected and preserved for (Díaz-Rodríguez *et al*. 2021). The utility of repurposing organisms for more than one use is highlighted by the fact that bacteria identified for one biological control or bioremediation trait often show additional phenotypes such as antibacterial or antifungal activities or plant growth promotion (Akbar *et al*. 2015).

Bacteriology research over decades has provided a wide range of tools to increase agricultural productivity. In the relationships between pest insects and crop plants, bacteria are a key part of the equation and can be used to promote plant health in various ways. Continuing to focus resources on microbial solutions will facilitate further development in this area for a sustainable future of agriculture.

ACKNOWLEDGEMENTS

Angel Guerrero received funding support from the National Summer Undergraduate Project. Lindsey Price Burbank is funded by the United States Department of Agriculture, Agricultural Research Service (USDA-ARS) appropriated project 2034-22000-014-000-D. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the USDA. The USDA is an equal opportunity provider and employer.

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細菌於農業害蟲防治的應用：過去、現在與未來

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摘要

Burbank, L. P. and A. Guerrero. 2025. Bacteria in insect pest management in agriculture: The past, present, and future. J. Taiwan Agric. Res. 74(4):377–397.

自古以來害蟲一直困擾著農業，農作物害蟲管理需隨之不斷創新。細菌與其他微生物透過發揮致病活性、產生有毒次級代謝物及提升植物防禦等能力，而成為防治害蟲的重要工具。此外，農業環境中的細菌通常具備代謝農業化學品的能力，故可用來減輕農業對環境的影響。然而，目前僅有小部分來自植物與昆蟲的細菌已被研究，還有大量的微生物多樣性尚未被運用。隨著對農業生產力需求的增加與氣候變遷改變病蟲害模式，探索更多具有害蟲生物防治與生物修復應用潛力的細菌種類至關重要。本綜述概述了細菌生物防治的一些歷史、細菌衍生農業化學品的使用、細菌導致殺蟲劑抗性以及目前對細菌-昆蟲-植物相互作用的理解。本文也進一步提出該領域的研究建議，以拓寬永續害蟲防治方案的範圍。

關鍵詞：殺蟲劑、生物防治、農業、有益細菌、害蟲管理。

投稿日期：2025 年 6 月 16 日；接受日期：2025 年 9 月 25 日。

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台灣農業研究 (J. Taiwan Agric. Res.) 74(4):399–414 (2025)
https://doi.org/10.6156/JTAR.202512_74(4).0003

Feature Article

Mini-Review on Microbial Pesticide Research for Crop Protection Assisted by Generative AI

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Abstract

Lazo, G. R., S. C. Chang, H. T. Shih, H. Huang, C. M. Wallis, A. Á. Pérez de León, and J. Chen. 2025. Mini-review on microbial pesticide research for crop protection assisted by generative AI. J. Taiwan Agric. Res. 74(4):399–414.

Research to develop safer pest control technologies is critical for modern agricultural crop protection. This need is heightened by public concern with drawbacks associated with the intense use of synthetic chemical pesticides resulting in human and non-target organism toxicity and pests resistant to agrochemical treatments. In contrast, microbial pesticides are environmentally friendly, and their use presents low risk for the development of resistance in target pest populations. Thousands of research publications on microbial pesticides accumulated since the 1950s and available in PDF (electronic format) could be analyzed to identify research areas to innovate microbial pesticides for sustainable pest control. However, searching through a large volume of research manuscripts is challenging for researchers due to limitations for exhaustive data mining and potential inaccuracies extracting massive science data. The use of Generative Artificial Intelligence (GenAI) tools could assist in reviews by facilitating the identification of publications across scientific disciplines with information that scientists can use to accelerate microbial pesticide research and innovation. This study employed a GenAI tool to assist reviewing 750 PDF publications on microbial pesticides collected from public databases. Summaries of relevant papers identified rapidly were queried by the co-authors with expertise in plant pathology and entomology to prepare a manuscript. Actual intelligence was practiced further by subjecting the manuscript to further evaluation and revision incorporating the co-authors' expertise emphasizing genomic research. Advantages and disadvantages of using GenAI technology in making literature reviews are discussed herein. This mini-review highlights the use of microbial pesticides in agriculture to provide long-term pest management options and documents how GenAI can assist actual intelligence to ideate agricultural research for sustainable crop protection.

Key words: Microbial pesticide, Generative artificial intelligence (GenAI), PDF publications, Bacterial biopesticides, Fungal biopesticides, Genomic.

Received: June 20, 2025; Accepted: September 25, 2025.

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INTRODUCTION

Pest management is crucial for producing crops to feed the world. Several species of invertebrate animals, including insects, mites, and nematodes, and microbes including fungi, bacteria, and viruses are considered pests because they damage and reduce the yields of crops. Other than viruses, crop pests are commonly controlled by chemical pesticides produced synthetically, or by biological pesticides also known as biopesticides. Chemical pesticides have been used intensely since the last century, which selects crop pest populations resistant to them. Additionally, the public is concerned with the pervasive use of chemical pesticides that can result in toxicity to human and non-target organisms, and pest resistance that render the pesticides ineffective. Thus, interest in research and development to use biopesticides as alternatives to chemical pesticides keeps growing (Thomas 1999; Ruiu 2018; van Lenteren *et al*. 2018; Keswani *et al*. 2019; Rani *et al*. 2021; da Silva Folli-Pereira *et al*. 2022; Elnahal *et al*. 2022; Mishra & Patni 2022; Thakur 2022; Ayilara *et al*. 2023; Aioub *et al*. 2024; Tadesse-Mawcha *et al*. 2024). There are three general types of biopesticides: (1) microbial biopesticides, consisting of microorganisms such as bacteria, fungi, viruses, and protozoa; (2) biochemical biopesticides or biorationals, derived from natural substances such as plant extracts and growth regulators; and (3) plant incorporated protectants, which are derived from genetically modified plants incorporated with genetic material that shows pesticide activity (EPA 2025) (https://www.epa.gov/ingredients-used-pesticide-products/what-are-biopesticides#classes).

Our program involves research and development of effective microbial pesticides including the discovery and characterization of bacteria and fungi that can be used as biopesticides for important crops. Microbial biopesticide research is documented in public domains and disseminated electronically in portable document format (PDF) files. Searches of scientific literature using PDFs could identify research areas to innovate microbial pesticides. However, searching through a large volume of research manuscripts is challenging for researchers due to limitations for exhaustive data mining and potential inaccuracies extracting massive science data. For example, a literature search we conducted using “microbial biopesticides” yielded a total of 5,045 entries in PubMed database of National Center for Biotechnology Information (NCBI) as of March 2025 (Fig. 1). And more specific searches using “bacterial biopesticides” yielded 8,660; “fungal biopesticides” yielded 11,190; “viral biopesticides yielded 227; and “nematode biopesticides” yielded 1,111 (Fig. 1). Interestingly, using “microbial” in the combined keyword yielded fewer entries than when using “bacterial” and “fungal” with “biopesticides”. This was likely due to the uncommon use of the term “microbial biopesticides” as a keyword in published literature, which reflected complexity that complicates comprehensive literature searches.

Searching through and summarizing a large volume of research papers is time-consuming and prone to human error that may result in synthesizing inaccurate information due to a large volume of data processing. Recent developments in artificial intelligence (AI) produced tools to automate electronic document reviewing processes (van Dijk *et al*. 2023; Fabiano *et al*. 2024; Ge *et al*. 2024). Large Language Models (LLMs) are a type of AI tool allowing the application of Generative Artificial Intelligence (GenAI) to assist literature reviews. Some LLMs may be accessed through subscription-serviced vendors such as OpenAI, Google, and Anthropic. However, there are a few that are available through publicly available resources such as huggingface.co and ollama.ai. Offering such as ollama.ai may be used offline if data security is a concern. Additionally, current public browsers such as Google Chrome and Microsoft Edge are equipped with AI functions that significantly improve the efficiency of internet searches using publicly available data.

This study explored how reviewing PDFs

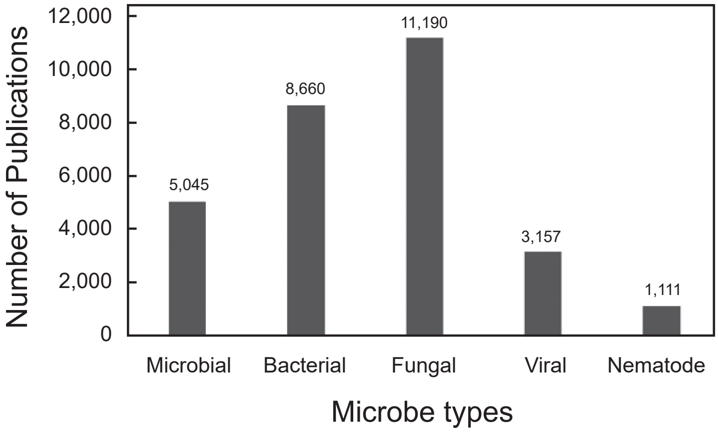

Fig. 1. Number of publications identified through search in PubMed database of National Center for Biotechnology Information (NCBI) using "microbial", "bacterial", "fungal", "viral", and "nematode" in combined keyword with "biopesticides" as of March 2025.

of scientific publications on microbial biopesticide research could be assisted by GenAI. Actual intelligence (AcI) based on our professional expertise was applied to curate and edit the draft version of this manuscript, which corrected output errors from the GenAI-aided literature search. Information was validated by closely reviewing selected references. Recent developments in genomics of biopesticide microorganisms and the research significance are highlighted. The assistance of GenAI technology in literature reviews is discussed.

PDF MANUSCRIPT DATA COLLECTION AND GENAI PROCEDURE

To test the use of GenAI procedure, 750 PDFs in the public domain were downloaded. A Langchain method was used with a retrieval augmented generation (RAG) protocol implemented to interface with an LLM. PDFs were parsed into chunks using an embedding tool (nomic-embed-text) and a vector store database (chromdb). A description of the process needed is available at the github repository (https://github.com/grlazo/AIRA/). Parameters of the vector store specified the chunksize to 800 with an overlap setting of 80. A variety of publicly available LLMs were screened via the ollama 0.5.7 (ollama.ai) framework. The LLM "Llama 3.18b" was chosen, which is in an 8 billion parameter trained model in a 4-bit quantized form with a 4K embedding length and 131K context window. Prompts retrieved the top 20 matches from the vector store and formulated a response based on the prompt presented to the LLM. Parameters can be adjusted for optimization of information received which in this case presented conditions apparently suitable for demonstration purposes.

Topics were resourced from subject matter experts and a series of prompts were presented and the answers scored (Table 1). The information retrieved was further evaluated, edited and updated based on responses available from public search engines including Google search, Microsoft Bing, and Meta AI. Selected PDFs were further examined by at least one coauthor. Corrections minimized possible hallucinations generated by AI. This enabled the application of AcI where humans correct GenAI outputs as needed. GenAI assistance to analyze and classify data adhered to guidance by the American Phytopathological Society (https://apsjournals.apsnet.org/page/authorinformation#AI).

Table 1. Initial topics and questions to sequester responses from GenAI based on 750 research publications related to microbial biopesticides.

Number	Topic	Questions
1	Historical perspective	“What is a microbial pesticide, and can you provide some historical perspective on how they came about?”
2	Economical and Academical Significance	“Why would microbial pesticides be used and are there any notable successful use cases?”
3	Biological natures of microbial biopesticides	“What types of microbial pesticides are in use and can you provide a ratio of the types that are currently in use?”
4	Bacterial pesticides	“Of the bacterial pesticides in use can you provide the mode of action and are there notable examples to be aware of?”
5	Fungal pesticides	“Of the fungal pesticides in use can you provide the mode of action and are there notable examples to be aware of?”
6	Viral pesticides	“Of the viral pesticides in use can you provide the mode of action and are there notable examples to be aware of?”
7	New technology	“Is there any mention of newer technologies in the use of microbial biopesticides in comparison to traditional application methods and can you provide some examples where success has been demonstrated?”
8	Microbial pesticides in genomic era	“Is there any mention of genome-based approaches which can be utilized for microbial biopesticide application, and can you provide examples?”

HISTORICAL PERSPECTIVE

The concept and practice of using microorganisms for pest control can be dated back to ancient times (Flint & van den Bosch 1981). Yet most documentational reports began in the 1800s. Ironically, many early works on microbial biopesticides started with pathogens on silkworm, a desirable insect. In 1835, an infectious disease of insect was observed by Agostino Bassi of Italy, in silkworm larvae caused by the fungus *Beauveria bassiana*. A Russian entomologist Elie Metchnikoff, reared *Metarhizium anisopliae*, a fungal pathogen, for suppression of the sugar beet curculio, *Cleonus punctiventris*, using fungal spores in 1884 (Hoddle & Van Driesche 2009). Both *B. bassiana* and *M. anisopliae* are still dominantly used fungal biopesticides nowadays. In 1911, the German scientist Berliner observed a disease of larvae of the flour moth, *Anagasta kuehniella*, and by 1938, a bacterium, *Bacillus thuringiensis*, was identified as the pathogen and being marketed for caterpillar pest control (Hoddle & Van Driesche 2009). These early works established the concepts of insect pathology, and, furthermore, procedures to rear the insect causative organisms that enabled the pest control practices.

As of March 2025, there are 446 microbial biopesticides registered by United States Environmental Protection Agency (EPA) (https://www.epa.gov/ingredients-used-pesticide-products/biopesticide-active-ingredients). The number of microbial biopesticide products is still in a trend of increasing mainly due to (1) regulators urging for sustainable plant protection, (2) food companies and consumers demanding zero-residue food, and (3) big agrochemical companies showing interest in biological alternatives as a new business opportunity (Preininger *et al*. 2018).

RELEVANCE AND ECONOMIC SIGNIFICANCE OF MICROBIAL BIOPESTICIDES

The justifications of using microbial biopesticides are that they are environmentally friendly, targeting only the intended pests but not beneficial insects or other natural enemies, and ecologically safer relative to chemical pesticides (Daraban *et al*. 2023). These are reflected

in target-specific range, delayed mode of action, reduced persistence, low residue levels, and safe in practice handling (Oguh *et al*. 2019). One key feature of microbial pesticides is that they are derived from natural sources, i.e. they are already in the environment (El-Wakeil *et al*. 2006; Daraban *et al*. 2023). For this reason, they are considered natural products, in contrast to the non-natural synthetic chemical pesticides. However, in all circumstances, it is a good practice that the time and manner of application of biopesticides should be kept in mind and the precautions that must be strictly observed.

Various microorganisms including fungi, bacteria, viruses, nematodes, and protozoa have been studied and tested for their efficacy against various plant pests and pathogens (Ruiu 2018; Kvakkestad *et al*. 2020). A rough estimate from our literature search shows that bacteria are the most used biopesticide type (60–70%), followed by fungi (20–30%). In line with our current research interests, this review focuses on bacterial and fungal biopesticides. It is also not intended to be a comprehensive literature review, but to highlight some basic perspectives of pesticidal microorganisms through selected examples. A particular interest is in microbial genomics, a more recently developed technology which has potential to revolutionize microbial biopesticide research and application. Reviews of other biopesticides such as viral biopesticides can be found in multiple publications (Ruiu 2018; Mondal *et al*. 2021; Tadesse-Mawcha *et al*. 2024).

BACTERIAL BIOPESTICIDES

Bacterial biopesticides have been used to control both insect/mite pests (Glare *et al*. 2017; Ruiu 2018; Gangwar *et al*. 2022; Tomar *et al*. 2024) and plant pathogens (Bonaterra *et al*. 2012; Glare *et al*. 2012; Boro *et al*. 2022). Over 200 bacterial species of bacteria have been tested for use as biopesticides. Most entomopathogenic bacteria belong to the Bacillaceae family (Alsaedi *et al*. 2017; Tadesse-Mawcha *et al*. 2024). Other groups of entomopathogenic bacteria include gammaproteobacteria such as the endosymbionts of insecticidal nematodes *Photorhabdus*, *Xenorhabdus*, *Serratia* species (Hurst *et al*. 2000; Akhtar *et al*. 2025), *Yersinia entomophaga* (Landsberg *et al*. 2011), and *Pseudomonas entomophila* (Vodovar *et al*. 2006); betaproteobacteria such as *Burkholderia rinojensis* (Cordova-Kreylos *et al*. 2013), *Chromobacterium subtsugae* (Martin *et al*. 2007); and alphaproteobacterial such as *Agrobacterium radiobacter* (McCardell & Pootjes 1976). Actinobacteria is also an important source of biopesticides. Different *Streptomyces* species produces a variety of insecticidal toxins, such as the macrocyclic lactone (Copping & Menn 2000; Diab *et al*. 2024), and *Saccharopolyspora spinosa* produces spinosins (Kirst 2010; Ruiu 2018; Kumar *et al*. 2021; Tadesse-Mawcha *et al*. 2024).

Bacillus thuringiensis is the most widely used bacterial biopesticides (Schnepf *et al*. 1998; Bel *et al*. 2024). As an insect pathogen, *B. thuringiensis* insecticidal activity is attributed to parasporal crystals, which are toxic to a wide variety of insect species among the orders Lepidoptera, Coleoptera, Hymenoptera, Diptera, Homoptera, Orthoptera, and Mallophaga, and against nematodes, mites, and protozoa (Schnepf *et al*. 1998). Once the insect larva consumes a crop leaf containing crytals, the protoxin is activated by gut alkaline pH by action of protease. The activated protein binds to gut wall receptor and causes permeation in hemocoel. The toxin dissolves in body cavity and lava dies in 24–48 h from septicemia leading to dead larvae (Rajamani & Negi 2021).

In the context of biocontrol of plant diseases, the three families of *Bacillus* lipopeptides- surfactins, iturins, and fengycins are known for their antagonistic activity for a wide range of potential phytopathogens such as *B. subtilis* to apple gray mold disease caused by *Botrytis cinerea* (Ongena *et al*. 2005) and *B. amyloliquefaciens* to *Fusarium oxysporum* f. sp. *cubense* (Yuan *et al*. 2012). Iturin and Fengycin have antifungal activities, while surfactin has broad range of potent antibacterial

activities (Meena & Kanwar 2015). Recent investigations have found that these lipopeptides can also influence the ecological fitness of the producing strain in root colonization and have a key role in the bacteria-plant interactions that stimulates host defense mechanisms (Ongena & Jacques 2008).

Many members of the *Pseudomonas fluorescens* complex are in fact known as effective biocontrol agents of plant pathogens and as plant growth promoters. For this reason, they are of great interest for biotechnological applications (Haas & Keel 2003; Haas & Défago 2005). The antagonistic activity of fluorescent *Pseudomonas* is mainly related to the production of several antibiotic compounds, lytic enzymes, lipopeptides, and siderophores. Several volatile organic compounds are also synthesized by fluorescent *Pseudomonas* including different kinds of molecules that are involved in antagonistic interactions with other organisms and in the induction of systemic responses in plants (García-Hidalgo *et al*. 2020; Raio 2024).

Agrobacterium radiobacter strains have been used to treat germinating seeds, roots and stems of stone fruit and nut trees for disease protection. *A. radiobacter* strain K84 occurs naturally in many types of soil. As a pesticide active ingredient, it is used in nurseries and greenhouses for controlling the closely related bacterium *A. tumefaciens*, which causes crown gall disease in plants due to the tumor-inducing (Ti) plasmid (McCardell & Pootjes 1976). *A. radiobacter* was formerly incorrectly synonymized with *A. tumefaciens* (Arahal *et al*. 2023). Unlike other members of its genus, it does not harbor a tumor-inducing (Ti) plasmid and is hence not plant pathogenic.

Bacterial pesticides demonstrate the diverse modes of action and their potential for large-scale commercialization. Their modes of action included (1) production of antibiotics that kill or inhibit the growth of plant pathogens; (2) direct interaction with plant pathogens, suppressing growth or reproduction; (3) competition with the pathogen for nutrients and niches in the host plant; (4) induction of host resistance, or the triggering of the plant's immune system to produce resistance against specific pathogens; and (5) plant growth promotion, or the enhancement of nutrient uptake to promote healthy growth and increase tolerance to stresses.

FUNGAL BIOPESTICIDES

Fungal biopesticides are commonly used to control insects, yet many fungi also work against other fungi, bacteria, and other plant pathogens (Leathers *et al*. 1993; Afshan 2023). Fungal biopesticides do not have to be ingested by target insects to cause infection. This is a distinct feature from bacterial and viral biopesticides. They can penetrate through the insect cuticle and cause infection either creating competition for space and nutrients or producing toxic secondary metabolites. Main fungal entomopathogens include species covering the following phyla: Chytridiomycota, Zygomycota, Oomycota, Ascomycota, and Deuteromycota (Ruiu 2018).

Beauveria bassiana is one of the most common fungal pesticides used to control insect pests. In taxonomy, *B. bassiana* is the anamorph (asexually reproducing form) of *Cordyceps bassiana*. The latter teleomorph (the sexually reproducing form) has been collected only in eastern Asia (Li *et al*. 2001). *B. bassiana* has been reported to control various insects, including termites, thrips, whiteflies, aphids and various beetles. In the disease cycle, the fungal spores/blastopores/conidia from insect killed by the fungus land on target insect, germinate through penetration peg. Inside the host hemocoel, fungi produce mycelium, blastopores and toxins. Mycelium inside host will germinate outward and kill the host (Tadesse-Mawcha *et al*. 2024).

Another popular fungal pesticide that has been used to control insects is *Metarhizium anisopliae* (Tang *et al*. 2019; Zekeya *et al*.

2022). One significant feature of the use of *M. anisopliae* is its compatibility with insecticides. In rice field, the fungus effectively infected two important planthoppers *Nilaparvata lugens* and *Sogatella furcifera*. In this case, *M. anisopliae* were used along with insecticides thiamethoxam and pymetrozine in field application. The combined use of *M. anisopliae* with insecticides resulted in more effective control of plant leafhoppers (Tang *et al*. 2019).

Members in the genus *Trichoderma* are noteworthy to mention for their extensive use as biopesticide against numerous aeronautical and soil-borne plant pathogens in field or greenhouse experiments (van Lenteren *et al*. 2018; Guzmán-Guzmán *et al*. 2023). Most biocontrol agents are from the species *T. asperellum*, *T. harzianum*, *T. viride*, and *T. hamatum*. In taxonomy, *Trichoderma* is the anamorphs of *Hypocrea*, an ascomycete. In general, *Trichoderma* spp. are non-plant pathogenic and opportunistic plant symbionts. The fungus establishes beneficial interactions with their hosts. However, many species of *Trichoderma* are pathogens of cultivated mushrooms refereed as “green mold” disease. Many fungal cell wall-degrading enzymes like chitinases, hydrolases, 1,3- proteases, glucanases, and mannanases are produced by different members of this genus (Thambugala *et al*. 2020; Saldaña-Mendoza *et al*. 2023). Other fungal species commercially exploited worldwide for pest management include *Verticillium lecanii*, *Lecanicillium* spp., *Hirsutella* spp., *Paecilomyces*, and *Isaria* spp. (Ruiu 2018).

Fungal pesticides are generally considered to be environmentally friendly in terms of low toxicity to mammals and birds. They can be applied through various methods, including spraying or immersing insects in fungal suspensions. The efficacy of fungal pesticides can be improved by optimizing application techniques, such as using the correct concentration and mode of delivery (Rajendran 2021).

MICROBIAL PESTICIDE RESEARCH AND DEVELOPMENT IN THE GENOMIC ERA

Various studies during the last century identified and characterized hundreds of bacterial and fungal species/strains with pesticidal properties from different hosts and regions around the world. However, conventional methodology often failed to reveal the genetic mechanisms of pesticidal activity, as well as many taxonomy issues among morphologically similar strains. Recent developments in next-generation DNA sequencing technology and genomics provided deeper insights into the genetic makeup of bio-pesticidal microorganisms. Two immediate and important impacts of genome sequencing discussed herein include: (1) accelerated identification of pathogenic genes and related regulatory factors; and (2) improved taxonomy systems for strain detection and selection through effective and accurate characterization of microbial populations.

Taking *B. thuringiensis* as an example. Since the first *B. thuringiensis* genome sequence was released in 2004 (Han *et al*. 2006), there are currently 1,410 genome sequences of this bacterium deposit in GenBank, including 105 complete and 5 metagenome-assembled genomes (MAGs). A full *B. thuringiensis* genome spans from 5.313 to 6.861 Mbp. The number of genes annotated varies from 5,343 to 7,227, and the number of plasmids ranges between 1 and 14. The guanine-cytosine content (GC) is between 31.4 and 35.48%. Analyses of these genome sequences confirm the vast genetic diversity among the studied strains. By exploring new strains, novel toxin genes and proteins have been detected (Jouzani *et al*. 2017; Pacheco *et al*. 2021; Yılmaz *et al*. 2025). An example genome of *B. thuringiensis* is shown in Table 2. The bacterial strain (IS5056) contains a circular chromosome of 5,491,935 bp and 14 plasmids ranging from 6,880 to 328,151 bp.

Table 2. List of sequenced genomes of selected bacteria and fungi used as active ingredient for microbial pesticide development.

Microbe names	Number of genome sequences	Bioactivity note	Example genomes	References
Bacteria				
Bacillus thuringiensis serovar *thuringiensis* (+ 16 other serovars)	1,410	Bt toxins against various insect pests	Strain IS5056, Chromosome: CP004123, 5,491,935 bp Plasmids: CP004137, pIS56-328, 328,151 CP004136, pIS56-285, 285,459 CP004135, pIS56-233, 233,730 CP004134, pIS56-107, 107,431 CP004133, pIS56-85, 85,134 CP004132, pIS56-68, 68,616 CP004131, pIS56-63, 63,864 CP004130, pIS56-39, 39,749 CP004129, pIS56-16, 16,206 CP004128, pIS56-15, 15,185 CP004127, pIS56-11, 11,331 CP004126, pIS56-9,9,671CP004125, pIS56-8, 8,251 CP004124, pIS56-6, 6,880 Total = 6,771,593 bp	Murawska *et al*. (2013)
Lysinibacillus sphaericus (*Bacillus sphaericus*)	65	Binary and mosquitocidal (Mtx) toxins	Strain DSM 28, CP019980.1, Chromosome, 4,681,723 bp	Lee *et al*. (2018)
Paenibacillus popilliae	2	Milky spore disease of the white grubs of Japanese beetles	ATCC 14706, ASM31523v1, chromosome (contig), 3,833,720 bp	Iiyama *et al*. (2023)
Agrobacterium radiobacter	27	Agrocin 84, a bacteriocin that inhibits the growth of *Agrobacterium tumefaciens* which causes crown gall disease	Strain LMG 267 CP169641.1, circular 2,974,514 bp CP169642.1, linear, 2,085,723 bp CP169643.1, pAtLMG267a 490,305 bp CP169644.1, pAtLMG267b 176,584 bp CP169645.1 pTiLMG267 194,263 bp Total = 5,921,389 bp	Weisberg *et al*. (2020)
B. subtilis	1,289	Antifungal and antibacterial lipopeptides	Strain 168, AL009126 4,215,606 bp	Kunst *et al*. (1997)
Pseudomonas fluorescens	321	Antibiotics and siderophores against fungal and nematode pathogens	Strain Pf0-1, CP000094, 6,438,405 bp	Silby *et al*. (2009)

Table 2. List of sequenced genomes of selected bacteria and fungi used as active ingredient for microbial pesticide development. (continued)

Microbe names	Number of genome sequences	Bioactivity note	Example genomes	References
Serratia marcescens	3,197	Antibiotics and siderophores against fungal pathogens	Strain ELP1.10 CP127881.1, Chr. 5,031,539 bp CP127882.1, pELP1.10 129,237 bp	Leung *et al*. (2023)
Fungi				
Beauveria bassiana	208	Toxins such as beauvericin, bassianin, bassianolide, beauverolides, tenellin, oosporein, oxalic acid, calcium oxalate crystals, and many beauvericin analogs	Strain HN6, Chr.1 CP045886.1; 6,424,025 bp Chr.2 CP045885.1; 5,597,631 bp Chr.3 CP045884.1; 5,431,745 bp Chr.4 CP045883.1; 4,613,348 bp Chr.5 CP045882.1; 3,702,357 bp Chr.6 CP045881.1; 3,525,630 bp Chr.7 CP045880.1; 3,028,184 bp Chr.8 CP045879.1; 1,996,242 bp Chr.9 CP045878.1; 1,357,674 bp Chr.10 CP045877.1; 615,663 bp Chr.11 CP045888.1; 454,462 bp Chr.12 CP045887.1; 387,253 bp Total = 37,134,214 bp	Guo *et al*. (2020) Wang *et al*. (2021)
Metarhizium anisopliae	9	Insecticidal cyclic peptides (destruxins).	Strain JEF-290, JABUBV; Chromosome: 42,848,098 bp	Lee *et al*. (2019)
Trichoderma harzianum	23	Antibiotics and cell wall-degrading enzymes	Strain BOL 12QD CP133624.1: Chr.1 CP133624.1; 7,677,970 bp Chr.2 CP133625.1; 7,232,126 bp Chr.3 CP133626.1; 6,421,825 bp Chr.4 CP133627.1; 4,804,947 bp Chr.5 CP133628.1; 4,726,887 bp Chr.6 CP133629.1; 4,458,692 bp Chr.7 CP133630.1; 4,296,277 bp Total = 39,618,724	Rocabado-Villegas *et al*. (2024)

Genomic studies on *B. thuringiensis* genomes have helped identify different genes encoding insecticidal proteins. The cry gene family, produced during the late exponential phase of growth in the bacterium, is a large and still-growing family of homologous genes. Each gene encodes a protein with strong specific activity against only one or a few insect species. The effectiveness and efficiency of genomic discoveries of insecticidal genes can be shown in the following example (Pacheco *et al*. 2021). In this work, the complete genome of *B. thuringiensis* strain GR007 that is toxic to *Spodoptera frugiperda* and *Manduca sexta* larvae were sequenced and annotated. Four replicons (one circular chromosome and three megaplasmids) were identified. The two largest megaplasmids (pGR340 and pGR157) contain multiple genes coding pesticidal proteins: 10 crystal (cry) toxin genes (cry1Ab, cry1Bb, cry1Da, cry1Fb, cry1Hb, cry1Id, cry1Ja, cry1Ka, cry1Nb, and cry2Ad), two vegetative insecticidal protein (vip) genes (vip3Af and vip3Ag), two binary toxin genes (vpa2Ac and vpb1Ca), five genes that codify for insecticidal toxin components (Tc's), and a truncated cry1Bd-like gene.

The whole genome sequencing fungal strains is more challenging because of the much larger genome size. With *B. bassiana* as an example, the first genome sequence was completed in 2012 with strain ARSEF 2860 (33.69 Mbp, 2 scaffolds). As of March 2025, there are 208 whole genome sequences submitted to the GenBank, compared to 1,410 genome sequences of *B. thuringiensis*. The full-length genome of *B. bassiana* spans from 28.68 to 38.31 Mbp with GC content between 48.5 and 53%. Of mentioning is the recent advancement of fungal genome sequencing at the resolution of chromosomal level as listed in Table 2. *B. bassiana* Strain HN6 was reported to have 12 chromosomes ranging from 387,253 to 6,424,025 bp with a total of 37,134,214 bp. The genome of *T. harzianum* Strain BOL 12QD harbors 7 chromosomes ranging from 4,296,277 to 7,677,970 bp with a total of 39,618,724 bp. It is expected that such chromosomal level sequencing will serve as significant references and genome structure frameworks facilitating genome research in both *B. bassiana* and *T. harzianum*, as well as other related fungi.

Another significant contribution of genome sequencing is the improvement of microbial taxonomy or systems for strain classification and identification. Taxonomy is critical for understanding the evolution of microorganisms and serves as the basis for evaluation of biocontrol agents. Accurate strain differentiation and collection are the first step towards identification of microbial strains with specific pest control properties. Genome sequencing techniques allowed for a more in-depth study of microorganisms with unclarified classification issues based on traditional methodologies such al morphology. For example, *B. bassiana* has long been used to describe a species complex of morphologically similar and closely related isolates. Recent molecular analyses indicated that *B. bassiana* consists of many distinct lineages that should be recognized as distinct phylogenetic species (Rehner & Buckley 2005; Rehner *et al*. 2011)

To evaluate the taxonomic status of *B. bassiana* with appropriate phylogenetic frameworks, whole genome sequences information was used in addition to morphological and secondary metabolites data, (Kobmoo *et al*. 2021). In this case, whole genome shotgun sequencing of 78 strains was performed. Clean reads were mapped to the *B. bassiana* reference genome ARSEF8028 (Valero-Jiménez *et al*. 2016). The selected single nucleotide polymorphisms (SNPs) (729,549 SNPs) from 1,132 genes were used for construction of phylogenetic tree. Three phylogenetic clades with high levels of divergence were found. Along with morphological data and secondary metabolite profiles, one clade remains to be *B. bassiana*, and two other clades were assigned to new species, *B. namnaoensis* and *B. neobassiana*.

The integration of genomics with microbial pesticide research holds great promise for research and discovering new microbial biopesticides. Future developments may include

(1) synthetic biology approaches to engineer microbes with custom pesticide properties; (2) CRISPR-based gene editing to enhance microbial effectiveness and specificity; (3) AI-driven genomic analysis to accelerate the discovery of new bio-pesticidal organisms; (4) metagenomic study by analyzing the genomes of soil and plant-associated microbes, new bacterial or fungal species with pesticidal properties can be discovered; and (5) investigation of RNA interference (RNAi) mechanism that could be used to produce a new generation of pesticides. By leveraging genomics, microbial pesticides can be optimized for efficiency, safety, and environmental sustainability, making them an effective and efficient tool for modern pest management.

CONCLUDING REMARKS

Public interests in microbial biopesticides as sustainable alternatives to chemical pesticides for agricultural pest management remains to be high in the years to come. The historical development of microbial biopesticides dates to ancient times and significant literature records started in the 1800s with landmark discoveries of *B. bassiana*, *M. anisopliae*, and *B. thuringiensis*. The relevance and economic significance of microbial biopesticides continues to benefit agriculture through their target specificity, reduced environmental persistence, and safety compared to chemical pesticides. These aspects are highlighted in the context of bacterial biopesticides such as *B. thuringiensis*, *Pseudomonas*, and *Agrobacterium*, and fungal biopesticides including *B. bassiana*, *M. anisopliae*, and *Trichoderma* species. Continued genomic research is expected to advance the development of technologies based on microbial biopesticides. Multi-omics including genomic approaches offer the opportunity to enhance our understanding of the diversity of microbes that could be used as pesticides by identifying pathogenic genes and finetuning the molecular taxonomy of bio-pesticidal bacteria and fungi.

Incorporating GenAI to screen publications in public databases facilitated the review process by identifying relevant PDFs from the voluminous scientific literature available electronically. However, this step occurring in the initial phase of the pursuit of knowledge with the assistance of GenAI resulted in mistakes that had to be corrected through AcI by the authors during the curation process. This interface of GenAI and AcI is critical for researchers to determine the reliability of what can be gained from topical literature reviews. Human oversight through AcI is fundamental to maximizing the benefit of using GenAI as a tool assisting in reviews like this one on microbial pesticide research for crop protection.

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生成式人工智慧輔助的作物保護微生物農藥研究簡述

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摘要

Lazo, G. R., S. C. Chang, H. T. Shih, H. Huang, C. M. Wallis, A. Á. Pérez de León, and J. Chen. 2025. Mini-review on microbial pesticide research for crop protection assisted by generative AI. J. Taiwan Agric. Res. 74(4):399–414.

開發更安全的害蟲防治技術對當前作物保護至關重要，公眾擔心大量使用合成化學農藥所產生的弊端加劇此一需求，此些弊端不僅會導致人類和非目標生物中毒，同時也會導致害蟲產生抗藥性。微生物農藥相較於合成化學農藥，對環境相對友善，且使用之後對目標害蟲族群產生抗藥性的風險也比較低。分析自 1950 年代以來累積的數千篇 PDF 格式 (電子格式) 的微生物農藥研究報告，可確認創新的微生物農藥是可以達到永續害蟲防治的研究領域。然而，受到能詳盡蒐尋資料的限制與提取巨量科學資料的潛在誤差，窮盡蒐尋大量研究文獻對研究人員而言，顯然是一項挑戰。使用生成式人工智慧 (GenAI) 工具可協助回顧並促進辨識跨學科的科學研究報告，為科學家提供可用於加速微生物農藥研究與創新的資訊。本研究使用 GenAI 工具輔助回顧從公共資料庫中所蒐集的 750 篇有關微生物農藥 PDF 格式報告，快速篩選出的相關論文摘要，經植物病理學與昆蟲學背景的共同作者檢視與歸納後，最終撰寫成稿。論文進一步運用實際智能，結合共同作者在基因體學研究的專業知識，對稿件進行進一步評估與修改，本文也討論使用 GenAI 技術進行文獻綜述的優缺點。本篇小型回顧報告著重介紹微生物農藥在農業的應用，以提供長期的蟲害管理方案，並闡述 GenAI 如何提供永續作物保護農業研究的實際智能構思。

關鍵詞：微生物農藥、生成式人工智慧 (GenAI)、PDF 出版品、細菌性生物農藥、真菌性生物農藥、基因組學。

投稿日期：2025 年 6 月 20 日；接受日期：2025 年 9 月 25 日。

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台灣農業研究 (J. Taiwan Agric. Res.) 74(4):415–447 (2025)
https://doi.org/10.6156/JTAR.202512_74(4).0004

Feature Article

Multiple Applications of Yellow Mealworm (*Tenebrio molitor* L.) Reared on Plant-Based Substrates: Circular Agriculture, Farmed Animal Feed, and Other High-Value Products

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Abstract

Hsieh, M. Y., W. B. Feng, P. C. Hsu, Y. J. Dong, Y. L. Lee, and H. T. Shih. 2025. Multiple applications of yellow mealworm (*Tenebrio molitor* L.) reared on plant-based substrates: Circular agriculture, farmed animal feed, and other high-value products. J. Taiwan Agric. Res. 74(4):415–447.

With the continuous growth of the global population, pressures on food supply and challenges in agricultural waste management have become increasingly severe. Insects have been increasingly recognized as a key solution to these issues. As biological agents capable of converting organic waste, insects can transform agricultural by-products and food processing residues (resources that are otherwise difficult to be used) into valuable outputs. This process not only promotes resource recycling and reduces the carbon footprint but also offers a sustainable alternative to conventional protein sources in animal feed, such as soybean meal and fishmeal, thereby supporting the sustainable development of both agriculture and animal husbandry. In the application of insects as feed ingredients for farmed animals, they provide high levels of protein and fat, making them a promising substitute for conventional animal feed components. Their cultivation requires fewer resources, has a short production cycle, and demands less space compared to traditional livestock, which positions insect farming as an efficient and environmentally friendly model in resource utilization. In addition to larval biomass, insect frass, the excreta and residual substrate generated during yellow mealworm rearing, constitutes an important by-product. Rich in nitrogen, phosphorus, chitin, and beneficial microorganisms, frass has shown potential as an organic fertilizer that enhances plant growth. Its application further reinforces the role of mealworm farming in sustainable waste recycling systems. This study takes the yellow mealworm (*Tenebrio molitor*) as a case study to explore its feasibility and advantages in decomposing plant-based residual materials and analyzes key considerations in its breeding process. In addition, this study assesses the potential of yellow mealworm larvae as a raw material in animal feed, including its nutritional value and practical application cases. It also reviews the prospects of using insect biomass and by-products in the development of high-value end products. The aim of this research is to provide a comprehensive understanding of the impact of resource insects on global food supply and environmental sustainability. Compared to traditional crop and livestock farming, insect farming exhibits

Received: July 8, 2025; Accepted: October 7, 2025.
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higher efficiency in resource utilization and significantly lower environmental impact, making it one of the green industries aligned with the United Nations Sustainable Development Goals (SDGs).

Key words: *Tenebrio molitor*, Plant-based substrates, Circular agriculture, Farmed animal feed, High-value applications.

INTRODUCTION

In 2022, the global population surpassed 8 billion, with projections indicating an increase to 10 billion by 2058 (Ritchie *et al*. n.d.). This rapid population growth has substantially heightened the global demand for crop production, as well as for food and livestock products (Verbeke *et al*. 2015). According to FAO (2024b), global production of crops, meat, eggs, and milk in 2022 reached 96, 361, 87, and 897 million tons, respectively, reflecting increases of 56%, 55%, 70%, and 61% compared to the year 2000. To meet the rising demand for animal-based proteins, the need for feed ingredients such as grains, soybean meal, fishmeal, and fish oil has also intensified. However, competition for limited arable land and water resources limits crop production for animal feed, further exacerbating food security challenges. Additionally, overfishing has restricted the availability of fishmeal, a critical component in livestock feed. These challenges underscore the urgent necessity to establish sustainable agricultural production systems, supported by relevant technologies and policy frameworks, which have become a top priority for governments worldwide.

FAO (2024a) emphasizes that reducing post-harvest losses is essential for enhancing the sustainability of food systems. According to UN (2020), approximately 13% of global food is lost between harvest and retail, while 19% of available food is wasted at the consumer level. In 2022, global food waste reached 105.2 million tons, primarily originating from food services, retail, and households (UNEP 2024). It is projected that by 2050, global waste generation will increase to 3.4 billion tons annually, representing a 70% increase of that in 2016, with 44% attributed to food waste and agricultural production residues (Kaza & Yao 2018).

Agricultural residues, also referred to as agricultural by-products, include leftover materials from agricultural production processes, such as rice straw, soybean meal, animal manure, and shrimp and crab shells. These residues are rich in cellulose, lignin, proteins, amino acids, minerals, and trace elements. However, conventional disposal methods, such as landfill burial and open field burning, contribute to greenhouse gas emissions (such as carbon dioxide, methane, and nitrous oxide), leading to air pollution and water contamination, which pose risks to ecosystems and human health (Koul *et al*. 2022).

One of the ultimate challenges of the twenty-first century is to ensure sufficient food production from healthy ecosystems for a growing global population. Establishing sustainable plant and animal production systems has become a critical strategic issue for global agriculture (Shih *et al*. 2021). A promising approach to this challenge involves incorporating insect species into agricultural production chains, utilizing them to decompose food waste and agricultural by-products, and converting them into economically valuable insect products such as animal feed, pet food, and industrial materials. This concept has emerged as one of the most popular topics in circular agriculture.

Insects are among the most ecologically diverse taxonomic groups, with a widespread global distribution. Stork (2018) notes that at least one million insect species have been formally described, with an estimated five million species yet to be discovered. Throughout Earth's long geological history, insects have continuously existed and evolved, playing a crucial role in shaping global biodiversity (Engel 2015). Different insect taxa occupy various ecological niches, providing numerous ecosystem services. These include roles

as decomposers (such as larvae of houseflies, flesh flies, black soldier fly (BSF), and scarab beetles), pollinators (such as adults of bees, bumblebees, and flower flies), herbivores (such as grasshoppers, larvae of butterfly and moth), predators (such as lacewings and mantids), and parasitic predators (such as parasitoid wasps). Additionally, insects serve as secondary producers, forming a crucial food source for insectivorous animals, engaging in symbiotic relationships with microorganisms, and acting as vectors for plant and animal pathogens (such as leafhoppers, aphids, planthoppers, and whiteflies).

The loss of insect biodiversity can result in a significant decline in global biodiversity. Approximately 75% of the world's crop species depend on insect pollination (Jankielsohn 2018; Wagner 2020). A reduction in insect diversity, especially pollinators, would pose a severe challenge to the survival of animals that depend on crops for sustenance. Therefore, insects are vital for maintaining biodiversity and ecosystem health, with the potential to contribute to agriculture, human and animal diets, medicine, and bioconversion processes. Their utilization aligns with the Sustainable Development Goals (SDGs) of United Nations (Barragán-Fonseca 2024).

This report reviews the global integration of insects into circular agricultural systems by using *Tenebrio molitor* Linnaeus (yellow mealworm, YM) as an example. The aim is to provide insights into the contributions of insect farming to animal nutrition and environmental sustainability by focusing on three major aspects: (1) the role of YMs in circular agriculture, (2) their applications in livestock farming, and (3) the emerging high-value uses of their biomass and by-products across various industries. Through mass breeding, insects are utilized as livestock feed, while their products and by-products are processed into materials of high-value end-product. Compared to crops or livestock, insect farming is a relatively energy-efficient and low-pollution, sustainable industry that aligns with the SDGs of United Nations. As for the application of insects in human food and pet food, this will be addressed in a separate review paper.

THE REASON FOR INSECTS TO SERVE AS A SUSTAINABLE FOOD SOURCE

Insects have long been an integral component of human diets across various cultures and geographical regions. Their nutritional composition is rich in protein, essential fatty acids, vitamins, minerals, and other bioactive compounds (Abdulsalam *et al*. 2019). Research indicates that early hominids used bone tools to collect and consume termites (Backwell & d'Errico 2001). Hardy *et al*. (2017) discovered 1.2-million-year-old fossils of *Homo* sp. from Spain, with dental calculus residues that contained insect fragments, plant fibers, meat, pollen, and fungal spores, providing direct evidence of entomophagy among early human ancestors. Additionally, historical records indicate that insect consumption was documented in China as early as 3,000 years ago (Chen *et al*. 2009). Insects for human food and medicine have been integral to cultural development throughout history. Although European cultures historically rejected entomophagy, Carl Linnaeus recorded that Europeans consumed larvae of bees, hornets, and ants in his zoological lectures as early as 1740 (Svanberg & Berggren 2021). Currently, entomophagy is practiced by over 2 billion people across 130 countries and more than 3,000 ethnic groups, incorporating between 1,000 and 2,200 insect species into human diets (Jongema 2017).

Beyond human consumption, insects also serve as a natural food source for many animals. Humans have developed animal feed formulas through long-term observation of the insect feeding habits of wild animals and semi-captive livestock. For instance, insects make up approximately 6% of the diet of wild cats (Veldkamp *et al*. 2012). Basuony *et al*.

(2005) reported that insects, including the American cockroach (*Periplaneta Americana* (Linnaeus)), beetles, grasshoppers, and mantises, were the second most abundant food component found in the stomachs of red foxes (*Vulpes vulpes* (Linnaeus)) in Egypt. Additionally, aquatic insects from Diptera, Trichoptera, Odonata, Hemiptera, and Coleoptera (both adult and larval stages), as well as terrestrial Hymenoptera insects from the families Vespidae (wasps) and Formicidae (ants), are common natural food sources for omnivorous and carnivorous fish. Fragments of insects from Hymenoptera, Diptera, and Coleoptera have also been identified in the digestive tracts of marine and saltwater fish (Goutham-Bharathi *et al*. 2013; Henry *et al*. 2015; Nogales-Mérida *et al*. 2019). Henry *et al*. (2015) reviewed studies on replacing fishmeal with insects in aquaculture feed, suggesting that interdisciplinary collaboration between entomologists and fish nutritionists is crucial to determine the optimal substitution ratio and the potential benefits of insects in improving fish health.

ADVANTAGES OF INSECTS IN CIRCULAR AGRICULTURE AND AS AN ALTERNATIVE PROTEIN SOURCE

Compared to conventional livestock, insects exhibit higher growth rates and reproductive capacities, enabling for stable production and mitigating seasonal fluctuations in crop and aquaculture supply. As poikilothermic animals, insects do not require additional metabolic energy for thermoregulation, enabling a higher proportion of energy intake to be allocated to growth. Consequently, insects demonstrate superior feed conversion rates (FCRs) compared to conventional livestock. The FCRs of commonly farmed insect species, such as *Acheta domesticus* (Linnaeus) (house cricket), *Hermetia illucens* (Linnaeus) (BSF), and *T. molitor* (YM), range from 1.50 to 2.08, which are comparable to aquaculture species (1.0–2.4) and broiler chickens (1.9) but significantly lower than that of pigs (2.7–5.0) and cattle (6.0–10.0) (Fry *et al*. 2018; Bawa *et al*. 2020; Deruytter & Coudron 2022).

Moreover, insects exhibit exceptionally high edible biomass yields, with nearly 100% of their body mass being consumable, in contrast to approximately 50% for conventional livestock, making them a highly efficient source of animal protein. From an environmental perspective, insect farming imposes a significantly lower ecological footprint compared to conventional livestock production, including reduced greenhouse gas emissions, lower land and water requirements, and improved energy efficiency (Lange & Nakamura 2021; Kim *et al*. 2022; Jafir *et al*. 2024). Moreover, insects can be reared on low-cost feed substrates, including agricultural by-products and food processing residues, with minimal space and operational costs compared to conventional meat production. This reduces reliance on soybean meal and fishmeal in livestock industries that contributes to the conservation of terrestrial and marine ecosystems while mitigating competition for food resources between human and livestock populations.

As a sustainable alternative protein and a key component of circular agriculture, insect farming aligns with multiple UN-SDGs, including SDG1 (No Poverty), SDG2 (Zero Hunger), SDG3 (Good Health and Well-being), SDG12 (Responsible Consumption and Production), SDG13 (Climate Action), SDG14 (Life Below Water), and SDG15 (Life on Land) (Dicke 2018).

INSECT SPECIES PERMITTED FOR ANIMAL FEED

Currently, 11 insect species have been permitted as feed sources for farmed animals worldwide (Table 1), spanning 4 orders and 6 families. These include 4 species from Orthoptera (*A. domesticus*, *Gryllus assimilis* (Fabricius), *Gryllodes sigillatus* (Walker), *Teleogryllus emma* (Ohmachi & Matsuura)), 2 species from Diptera (*Musca domestica* Linnaeus, *H. illucens*), 1

Table 1. Insects species permitted as animal feed in the world.

Species	EU[z]	UK[y]	Canada[x]	USA[w]	China[v]	Taiwan[u]	Korea[t]	Japan[s]	African[r]
Orthoptera									
Gryllidae									
Acheta domesticus, house cricket	✓	✓							
Gryllus assimilis, jamaican field cricket	✓	✓							
Gryllodes sigillatus, tropical house cricket	✓	✓							
Teleogryllus emma, oriental garden cricket							✓		
Diptera									
Muscidae									
Musca domestica, common housefly	✓	✓						✓	
Stratiomyidae									
Hermetia illucens, black soldier fly (BSF)	✓	✓	✓	✓		✓	✓		✓
Hymenoptera									
Apidae									
Apis mellifera, western honey bee						✓			
Coleoptera									
Tenebrionidae									
Alphitobius diaperinus, lesser mealworm	✓	✓							
T. molitor, yellow mealworm (YML)	✓	✓			✓	✓	✓	✓	
Zophobas morio, superworm						✓			

Table 1. Insects species permitted as animal feed in the world. (continued)

Species	EU[z]	UK[y]	Canada[x]	USA[w]	China[v]	Taiwan[u]	Korea[t]	Japan[s]	African[r]
Lepidoptera									
Bombycidae									
Bombyx mori, domestic silk moth	✓				✓	✓		✓	

[z] Information collected from Jensen *et al*. (2021).
[y] Information collected from Tiwasing & Pate (2024).
[x] Information collected from Larouche *et al*. (2023) and NPC (2022).
[w] Information collected from Morrison Foerster (https://www.mofo.com/resources/insights/211028-alternative-protein-industry-series, visit on 3/27/2025). The Association of American Feed Control Officials (AAFCO) has approved BSF larvae for animal feed.
[v] Information collected from website of Ministry of Agriculture and Rural Affairs, People's Republic of China (http://www.moa.gov.cn/hd/zqyj/201203/P020120307371176222741.doc, visiting on 3/27/2025). Insects (including but not limited to silkworm pupa meal, defatted silkworm pupa, and mealworm meal) may be processed and incorporated into animal feed, provided they do not pose risks to public or animal health.
[u] Information collected from Lee *et al*. (2021).
[t] Information collected from website of Ministry of Agriculture, Food and Rural Affairs, Korean. (https://www.mafra.go.kr/home/5109/subview.do?enc=Zm5jdDF8QEB8JTJGYmJzJTJ-GaG9tZSUyRjc5MiUyRjU2MzkwNSUyRmFydGNsVmlldy5kbyUzRg%3D%3D, visiting on 3/27/2025).
[s] Information collected from website of Research Institute of Environment, Agriculture and Fisheries, Osaka Prefecture (https://www.knsk-osaka.jp/ibpf/guideline/, visiting on 3/27/2025)
[r] Information from Grabowski *et al*. (2020). Regulatory frameworks regarding the inclusion of insects in animal feed vary across countries, with many lacking specific guidelines on their use. In some countries, such as South Africa and Niger, the farming of black soldier flies for animal feed production is legally permitted.

species from Hymenoptera (*Apis mellifera* Linnaeus), 3 species from Coleoptera (*Alphitobius diaperinus* (Panzer)), *T. molitor*, *Zophobas morio* (Fabricius)), and 1 species from Lepidoptera (*Bombyx mori* Linnaeus).

Among these species, *T. molitor* is the second most widely approved specie after *H. illucens*, having been authorized in 6 countries or economic regions, including the EU, UK, China, Taiwan, South Korea, and Japan. Additionally, within the three approved species from the Tenebrionidae family, *T. molitor* has the broadest regulatory acceptance, underscoring its high industrial value and global recognition.

YM (*T. molitor*) CHARACTERISTICS

YM is one of the few insect species currently utilized in circular agriculture, animal feed, and high-value products. Taxonomically, it belongs to the class Insecta, order Coleoptera, and family Tenebrionidae. As a holometabolic insect, its life cycle includes 4 stages: egg (1–4 wk), larva (8–12 wk), pupa (1–4 wk), and adult (3–6 mo) (Fig. 1). YM primarily inhabits warm, humid, and dark environments, with optimal growth conditions at 25–28℃ and relative humidity (RH) of ≥ 70% (optimal RH range: 60–75%) (Barrett *et al*. 2023; Parsa *et al*. 2023). The eggs are glossy milky-white, approximately 1.2–1.4 mm in length. Newly hatched larvae are also milky-white but gradually turn yellowish-brown as they develop, undergoing 7–20 instars and reaching a final length of approximately 28–32 mm. Pupae are light yellowish-brown and measure around 15–17 mm, while adults are dark reddish-brown, ranging from 14–19 mm in length. Newly emerged adults exhibit a lighter coloration, are nocturnal, and lay eggs either individually or in clusters.

YM is believed to have originated from the Mediterranean region. However, it is now widely distributed due to the global transportation and trade of stored products (Moruzzo

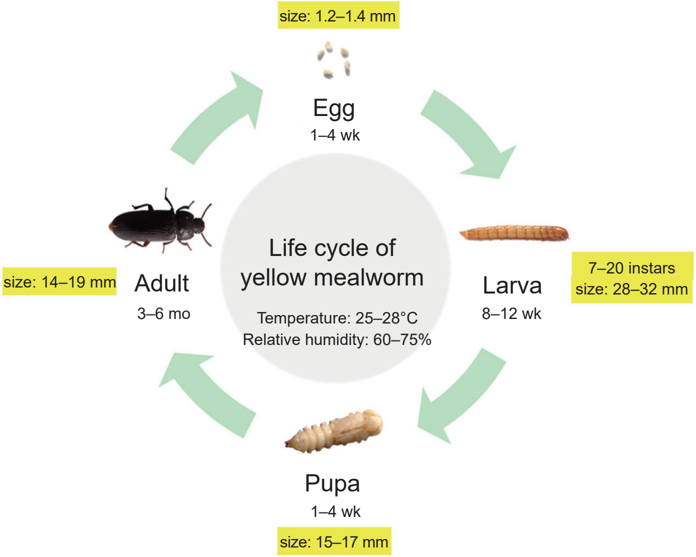

Fig. 1. Life history of *Tenebrio molitor*.

et al. 2021). It is classified as a secondary stored-product pest, capable of infesting flour, wheat bran, bread, and other processed cereal products, particularly under suboptimal storage conditions. However, improved global storage management has led to a significant decline in infestations in recent years.

Due to the high nutritional value of larvae and their adaptability to diverse feed substrates and rearing conditions (Bordiean *et al*. 2022), as well as their higher nutritional content compared to pupae and adults (Khanal *et al*. 2023), there have been approximately 300 billion yellow mealworm larvae (YML) reared annually worldwide as feed for amphibians, reptiles, and birds or processed into raw material for aquaculture, livestock feed (such as poultry, pigs, fish), and pet food. Additionally, YML has also been incorporated into circular agriculture systems and even human food production (Barrett *et al*. 2023). Currently, *T. molitor*, along with *A. diaperinus* and *A. domesticu*, is legally approved by the EU for use in animal feed, pet food, and human consumption (Commission Regulation (EU) 2017/893, 2017/2470, 2021/1372, 2022/188, 2023/58).

DEVELOPMENT OF YM APPLICATIONS IN CIRCULAR AGRICULTURE - LIMITED TO REARING ON AGRICULTURAL RESIDUE SUBSTRATES

Feasibility analysis based on rearing research cases, impacts of the feed nutritional composition

YML are highly adaptable to the decomposition of agricultural residues, making them one of the key insects of interest in circular agriculture (Nava *et al*. 2020). Numerous studies have explored the use of various agricultural production residues as rearing substrates for YML, including spent mushroom substrate (SMS), vegetable and fruit peels, olive pomace, rapeseed meal, sunflower seed meal, by-products of seed cleaning process of legumes (such as vetch, pea, lupin, lentil), beer yeast, cereal cultivation residues (such as rice straw, corn stalks), and cereal processing by-products (such as rice bran, rice husks, brewer's grain, black

wheat, barley, durum wheat, oats, corn, triticale) (Kim *et al*. 2014; Oonincx *et al*. 2015; van Broekhoven *et al*. 2015; Li *et al*. 2020; Rumbos *et al*. 2021; Bordiean *et al*. 2022; Vrontaki *et al*. 2024).

Although YML can utilize a broad spectrum of agricultural residues, their adaptability and growth performance vary depending on substrate composition. Compared to BSF, YM primarily thrives on dry substrates, only requiring moisture from a few vegetables or fruit (such as market rejects) as supplementary sources. Suitable substrates for YM have a lower moisture content, whereas BSF larvae grow better on agricultural residues with moisture content above 60% (Kröncke & Benning 2022; Barrett *et al*. 2023), making them more suitable for semi-solid to liquid substrates.

Current studies indicate that although YM can digest various agricultural residues, their growth performance remains suboptimal compared to traditional rearing substrates such as wheat bran and carrots. Fully replacing traditional substrates with plant-based agricultural residues has proven challenging. Cereal processing by-products, however, show potential to entirely replace wheat bran as a rearing substrate for YML. Despite this, YML reared on cereal by-products show slightly lower survival rates, larval weight, and pre-adult development time compared to those reared on wheat bran (Oonincx *et al*. 2015; van Broekhoven *et al*. 2015; Rumbos *et al*. 2021). Other agricultural residues are unlikely to serve as a complete substitute for wheat bran.

For homeothermic animals, total feed energy content directly impacts growth rate and efficiency, but as poikilothermic animals, YM does not expend additional energy to maintain body temperature, making the nutritional composition of the feed more important than its total caloric content. Protein content plays a crucial role in the development and survival of YML. Low-protein diets are associated with reduced larval survival and prolonged developmental periods, whereas high-protein diets accelerate development, enhance feeding activity, and promote larval weight gain. (Oonincx *et al*. 2015; van Broekhoven *et al*. 2015). However, the relationship between protein content and larval growth is not always linear, and other nutritional factors (such as fat, amino acids, vitamins, and minerals) also play the roles (Rumbos *et al*. 2021). Additionally, as a stored-product pest, growth of YML remains dependent on carbohydrate-rich substrates like cereals. Therefore, when developing alternative diets, careful consideration must be given to their nutritional composition, ensuring an optimal carbohydrate-to-protein ratio to mitigate risks of malnutrition, suboptimal growth, and potential cannibalism among larvae.

In addition to adjusting the nutritional composition of the rearing substrate, it is also important to note that different insect species have varying abilities to adapt to feed substrates. Some researchers propose a staged decomposition approach, utilizing different insect species for processing agricultural residues. Wang *et al*. (2017) found that corn stalks pre-degraded by YML are better suited for consumption by BSF larvae, leading to higher biomass production. This approach could also be extended to agricultural residues that are less suitable for YML rearing, thereby improving their utilization feasibility and enhancing the overall bioconversion efficiency of these waste materials.

Advantages based on rearing costs and avoidance of animal diseases

With the increasing commercialization and large-scale production of YML, breeding costs have become a key issue for enterprises worldwide. Traditional YML farming primarily depends on wheat bran as a carbohydrate source, supplemented with vegetables such as carrots, cabbage, or potatoes for moisture. However, these feed substrates are also human food, raising concerns that the production of YML as an alternative protein source may intensify the competition for food between humans, livestock, and pets. Therefore, identifying suitable alternative feed substrates to replace conventional feed formulations has become a critical research focus.

Agricultural residues generated during crop cultivation or processing offer a potential solution to the problem of food competition and reduce breeding costs associated with large-scale YML farming. By incorporating locally sourced agricultural by-products into YML feed formulations, can effectively lower production costs while facilitating the processing and utilization of agricultural wastes, leading to a decrease in market product prices, increased competitiveness, and improved economic feasibility.

Additionally, insects lack genes encoding prion proteins, which are responsible for transmissible spongiform encephalopathy (TSE) in herbivorous animals (Gałęcki *et al*. 2023). If insects are fed with plant-based substrates, the risk of spreading animal diseases can be effectively avoided, ensuring the safety and reliability of YML as ingredients in the animal feed and pet food.

Most importantly, this strategy facilitates the integration of YML into the circular economy model, enabling the continuous recycling and efficient utilization of agricultural resources. This further reinforces the role of YML in circular agriculture, contributing to the realization of sustainable development goals.

Important issues to be considered in rearing YML: Pesticide, heavy metal, and toxic residue contamination

Previous studies have demonstrated that YML can utilize a broad range of feed substrates, as well as possessing the capacity of degrading and metabolizing certain toxic compounds (Siddiqui *et al*. 2024). However, the potential presence of harmful contaminants- such as pesticides, heavy metals, plastic microparticles, pathogenic microorganisms, and mycotoxins-must be investigated in plant-based substrates used for feed. It is essential to prevent these substances from entering the food chain through insect biomass.

Houbraken *et al*. (2016) reported that pesticides can accumulate in YML bodies through feeding on contaminated substrates, particularly those with high n-octanol-water partition coefficients (Kow), which are readily absorbed and bioaccumulated in insects. Mlček *et al*. (2017) investigated the accumulation of heavy metals (Pb, Cd, Cu, Zn) in YML exposed to contaminated feed substrates and found that YML exhibited a higher absorption and retention capacity for copper (Cu) and zinc (Zn). Additionally, if the feed substrate contains materials with incomplete removal of plastic packaging, residual microplastics such as polyethylene (PE), polypropylene (PP), or polyvinyl chloride (PVC) may persist and accumulate in the YML, potentially leading to stunted growth and reduced survival rates (Peng *et al*. 2023).

During storage, agricultural residues may serve as reservoirs for harmful microorganisms (such as bacteria: *Salmonella* spp., *Listeria* spp., enteropathogenic *Escherichia coli* (Migula), *Clostridium perfringens* (Veillon & Zuber)), as well as mycotoxins (such as aflatoxin, vomitoxin, fumonisins, zearalenone, ochratoxin, T-2 toxin). Yet, studies have shown that YML can metabolize ingested aflatoxin B1 and zearalenone into less toxic derivatives or excrete them directly. Implementing a fasting period of at least one day prior to harvest can further reduce residual toxin levels, ensuring compliance with safety standards (Bosch *et al*. 2017; Niermans *et al*. 2019; Zhao *et al*. 2022). Still, some mycotoxins, such as T-2 and HT-2 toxins produced by *Fusarium* spp. in contaminated grains, may be accumulated in YML tissues (Piacenza *et al*. 2021), that raises the concerns regarding their suitability as feed ingredients for livestock farming and aquaculture.

Beyond pollutant residues, certain plants naturally contain antinutritional factors or alkaloids such as saponins, may adversely affect insect growth and development, thereby influencing the safety and efficacy of YML as an ingredient in animal feed and pet food (Schrögel & Wätjen 2019). Therefore, rigorous evaluation of agricultural residues used as feed substrates for YML rearing is essential to ensure the absence of hazardous contaminants, verify non-toxicity

to the larvae, and establish a reliable and sustainable supply chain. Meeting these criteria is crucial to ensure the use of YML reared on agricultural by-products as a cost-effective and stable protein source. This approach not only enhances their application in the animal feed and pet food industries but also reinforces circular economy principles that support sustainable production systems.

DEVELOPMENT OF YM APPLICATIONS IN LIVESTOCK FARMING INCLUDING POULTRY, LIVESTOCK, AND AQUATIC ANIMALS

Nutritional value of YML

YML is composed of crude protein (37.3–76.2%), crude fat (6.1–58.2%), crude fiber (3.5–38.8%), and ash (2.6–8.1%) on a dry matter (DM) basis (Table 2), with substantial variation influenced by the rearing substrate and environmental conditions. The crude protein content of YML is comparable to that of house crickets and fishmeal, and significantly exceeds that of BSF larvae and soybean meal. Similarly, crude fat and crude fiber contents in YML exceed those in house crickets, BSF larvae, soybean meal, and fishmeal, further reinforcing their potential as a high-quality alternative protein source for animal feed.

YML are rich in various amino acids (Table 3), particularly glutamic acid (Glu, 2.08–12.26% DM), leucine (Leu, 1.39–10.90% DM), lysine (Lys, 1.20–10.56% DM), and alanine (Ala, 2.48–10.09% DM). Other notable amino acids include proline (Pro, 1.60–9.59% DM), asparagine (Asn, 1.54–8.51% DM), phenylalanine (Phe, 1.20–8.20% DM), tyrosine (Tyr, 2.15–8.15% DM), valine (Val, 1.89–7.61% DM), and threonine (Thr, 1.20–7.50% DM), whereas cysteine (Cys, 0.35–3.16% DM) and methionine (Met, 0.52–2.52% DM) are present in lower concentrations. Compared to soybean meal, fishmeal, and other insect species, YML

Table 2. Main constituents (%) of *Tenebrio molitor* compared to other protein sources.

Main constituents	*Acheta domesticus*[z] House cricket	*Hermetia illucens*[y] Black soldier fly larvae (BSF)	*T. molitor*[x] Yellow mealworm larvae (YML)	Soybean meal[w]	Fish meal[v]
Dry matter (DM)	21.1–47.7	21.9–98.9	31.1–99.0	86.9–92.8	92.1
Crude protein (CP, %DM)	15.4–76.2	27.5–65.5	37.3–76.2	41.8–50.0	65.3–72.3
Crude fat (CF, %DM)	3.3–43.9	4.6–51.5	6.1–58.2	0.8–1.4	10.0–10.4
Crude fiber (CF, %DM)	3.7–10.2	4.1–23.2	3.5–38.8	3.4–7.9	0.3–0.8
Ash (%DM)	3.0–11.5	2.7–19.7	2.6–8.1	5.6–7.9	17.2
Chitin (%DM)	6.1–8.3	3.9–6.7	4.3–8.9	NR[u]	NR

[z] Data compiled from Pilco-Romero *et al.* (2023), Ververis *et al.* (2022), Magara *et al.* (2021), Udomsil *et al.* (2019) and Nogales-Mérida *et al.* (2019).
[y] Data compiled from Vasilopoulos *et al.* (2024), Lu *et al.* (2022), Zulkifli *et al.* (2022), Abd El-Hack *et al.* (2020) and Nogales-Mérida *et al.* (2019).
[x] Data compiled from Vasilopoulos *et al.* (2024), Syahrulawal *et al.* 2023, Shafique *et al.* (2021), Hong *et al.* (2020) and Nogales-Mérida *et al.* (2019).
[w] Data compiled from Lu *et al.* (2022), Abd El-Hack *et al.* (2020), Ibáñez *et al.* (2020), Lagos & Stein (2017) and Hussein *et al.* (2017).
[v] Data compiled from Lu *et al.* (2022), Abd El-Hack *et al.* (2020) and Hussein *et al.* (2017).
[u] NR: not reported.

Table 3. The amino acid compositions of *Tenebrio molitor* compared to other protein sources.

Amino acids (%DM[z])	*Acheta domesticus*[y] House cricket	*Hermetia illucens*[x] Black soldier fly larvae (BSF)	*T. molitor*[w] Yellow mealworm larvae (YML)	Soybean meal[v]	Fish meal[u]
Essential amino acid (EAA)					
Arginine (Arg)[t]	3.92–6.10	1.73–6.20	1.80–6.14	2.30–3.60	2.85–4.20
Cysteine (Cys)	0.40–0.80	0.01–1.38	0.35–3.16	0.60–0.80	0.38–0.70
Histidine (His)	1.32–2.25	0.77–3.25	0.84–4.44	1.25–1.41	0.92–1.50
Isoleucine (Ile)	0.04–4.45	0.13–4.80	1.07–6.48	1.00–2.50	1.63–3.20
Leucine (Leu)	0.07–9.75	0.19–7.83	1.39–10.90	3.33–3.90	2.95–4.80
Lysine (Lys)	0.05–5.40	0.15–7.40	1.20–10.56	1.00–3.12	2.27–5.50
Methionine (Met)	0.01–1.40	0.05–3.72	0.52–2.52	0.60–0.70	0.44–2.20
Phenylalanine (Phe)	0.03–3.00	0.07–7.76	1.20–8.20	2.20–2.60	1.99–2.60
Threonine (Thr)	0.74–3.60	0.20–4.50	1.20–7.50	1.10–2.60	1.51–3.10
Tryptophan (Trp)	0.13–0.55	0.02–0.70	0.02–5.80	0.65–1.20	0.39–0.70
Tyrosine (Tyr)	1.00–4.44	1.71–6.71	2.15–8.15	1.49–1.90	1.42–2.00
Valine (Val)	0.05–4.50	0.03–6.79	1.89–7.61	1.60–2.40	1.72–3.90
Non-essential amino acid (non-EAA)					
Alanine (Ala)	3.67–8.85	1.40–8.21	2.48–10.09	1.84–2.20	1.90–4.20
Asparagine (Asn)	NR[z]	NR	1.54–8.51	NR	NR
Aspartate (Asp)	4.73–7.75	3.22–5.28	3.07–4.89	4.82–5.50	4.63–5.80
Asparagine (Asn) + Aspartate (Asp)	4.61	NR	0.50–9.74	NR	NR
Glutamate (Glu)	6.87–10.45	0.85–6.37	2.08–12.26	7.40–8.90	8.14–8.40
Glutamine (Gln)	NR	NR	NR	NR	NR
Glutamate (Glu) + Glutamine (Gln)	6.45	3.84–13.10	3.80–12.53	NR	NR
Glycine (Gly)	1.04–4.27	0.12–6.15	1.30–6.60	1.81–4.30	1.73–5.00
Proline (Pro)	1.15–4.31	2.14–6.68	1.60–9.59	2.07–2.80	2.21–3.10
Serine (Ser)	1.02–3.02	1.50–4.88	1.36–6.60	1.93–2.40	2.07–2.60

[z] DM: dry matter; NR: not reported.
[y] Data compiled from Pilco-Romero *et al*. (2023), Magara *et al*. (2021), Udomsil *et al*. (2019) and Nogales-Mérida *et al*. (2019).
[x] Data compiled from Vasilopoulos *et al*. (2024), Lu *et al*. (2022), Zulkifli *et al*. (2022), Abd El-Hack *et al*. (2020) and Nogales-Mérida *et al*. (2019).
[w] Data compiled from Vasilopoulos *et al*. (2024), Syahrulawal *et al*. 2023, Shafique *et al*. (2021), Hong *et al*. (2020) and Nogales-Mérida *et al*. (2019).
[v] Data compiled from Lu *et al*. (2022), Abd El-Hack *et al*. (2020), Lagos & Stein (2017) and Hussein *et al*. (2017).
[u] Data compiled from Lu *et al*. (2022), Abd El-Hack *et al*. (2020) and Hussein *et al*. (2017).
[t] Arg is not essential for most animals, it is considered essential for birds and certain mammals (cats and ferrets).

contain higher levels of most amino acids, except for methionine and aspartic acid. Notably, YML are a rare source of asparagine, an absent amino acid in soybean meal, fishmeal, house crickets, and BSF larvae. In addition to supplying all 11 essential amino acids (EAAs) required by animals, YML also provide arginine (Arg), an EAA for birds and certain mammals (cats and ferrets). The EAAs in YML have been shown to enhance meat quality and feed efficiency in farmed animals (Hou & Wu 2018; An *et al*. 2025). The substantial presence of non-essential amino acids (NEAAs) in YML further reduce the metabolic energy required for protein synthesis in animals, thereby improving feed efficiency and reinforcing the suitability of YML as a sustainable protein source.

YML are rich in unsaturated fatty acids (UFAs) (Table 4), with monounsaturated fatty acids (MUFAs) being the most predominant, accounting for 8.01–59.39% DM. Oleic acid (C18:1n9) is the principal MUFA, comprising 3.55–57.63% DM. The polyunsaturated fatty acid (PUFA) content in YML ranges from 7.24–33.70% DM, with linoleic acid (C18:2n6) being the predominant PUFAs (8.01–35.58% DM). However, YML contain relatively low levels of omega-3 (n-3) fatty acids (0.11–1.80%, DM), resulting in a higher n-6/n-3 ratio (0.69–71.42). The saturated fatty acid (SFA) contents in YML are lower than those of BSF larvae, but comparable to t those of house crickets, with values ranging from 4.94% to 35.40% of DM. Among SFAs, palmitic acid (C16:0) is the most abundant component, followed by stearic acid (C18:0), pentadecylic acid (C15:0), and myristic acid (C14:0). Notably, YML lack lauric acid (C12:0), a fatty acid that is abundant in BSF larvae and recognized for its antimicrobial properties While YML have a high UFA content, their low omega-3 levels and elevated n-6/n-3 ratio limit their suitability in animal feed (Oonincx *et al*. 2015). Therefore, while YML serve as a viable protein source for livestock, their application in animal feed remains suboptimal unless dietary modifications are implemented to optimize their fatty acid profile.

The mineral composition of YML is presented in Table 5. Potassium (K) (3,737–19,290 mg kg^{-1} DM), magnesium (Mg) (200–16,300 mg kg^{-1} DM), and phosphorus (P) (700–14,290 mg kg^{-1} DM) are the predominant minerals, followed by calcium (Ca) (168–5,000 mg kg^{-1} DM) and sodium (Na) (404–3,644 mg kg^{-1} DM). Compared to house crickets and BSF larvae, YML contain higher P and Mg but lower Ca and Na than BSF larvae. Relative to soybean meal and fishmeal, YML exhibit comparable P and Mg levels but lower Ca and Na levels. Additionally, YML contain trace amounts of heavy metals, including manganese (Mn), Zn, Cu, and iron (Fe), with concentrations below 200 mg kg^{-1} (DM). These levels are comparable to those found in house crickets, soybean meal, and fishmeal. In contrast, BSF larvae tend to bioaccumulate higher concentration of heavy metals from food substrates. Studies indicate that BSF larvae can bioaccumulate Mn, Zn, Cu, and Fe at levels reaching 166, 103, 10.7, and 191 g kg^{-1} (DM), respectively, substantially higher than those found in YML, house crickets, soybean meal, and fishmeal. However, this does not imply that YML are entirely devoid of the risk of heavy metal accumulation. Bednarska & Świątek (2016) demonstrated that when YML were fed with Cd-containing flour, the concentration of cadmium (Cd) in their tissues increased with the amount consumed and the duration of exposure, indicating a potential risk of heavy metal accumulation. Overall, YML serve as a valuable source of minerals and can effectively replace soybean meal as a mineral supplement in animal feed. However, additional Ca and P supplementation should be considered when replacing fishmeal.

In addition to their major nutrients, YML contain bioactive compounds that may contribute to animal health, including vitamins (B12, B3, B2, B5, B7, E, and H) and bioactive substances such as antimicrobial peptides (AMPs), polysaccharides, and β-glucanase (Moruzzo *et al*. 2021; Syahrulawal *et al*. 2023). AMPs, including α-helix peptides, defensins, and

Table 4. The fatty acid compositions of *Tenebrio molitor* compared to other protein sources.

Fatty acids (%DM[z])	*Acheta domesticus*[y] House cricket	*Hermetia illucens*[x] Black soldier fly larvae (BSF)	*T. molitor*[w] Yellow mealworm larvae (YML)
Saturated fatty acid (SFA)	3.14–32.36	36.20–81.81	4.94–35.40
C_{10}:0, Capric acid	0.01	0.00–2.03	NR[z]
C_{12}:0, Lauric acid	0.02–0.27	7.5–65.1	0.01–0.38
C_{14}:0, Myristic acid	0.04–1.65	5.00–11.77	0.20–5.21
C_{14}:1n5, Myristoleic acid	0.02–0.44	NR	NR
C_{15}:0, Pentadecylic acid	0.01–0.11	0.13–14.38	0.06–7.10
C_{16}:0, Palmitic acid	1.56–24.81	1.03–24.59	3.43–23.60
C_{17}:0, Margaric acid	0.02–0.12	NR	NR
C_{17}:1, Civetic acid	0.24	NR	NR
C_{18}:0, Stearic acid	0.58–8.54	0.98–6.90	0.07–7.92
C_{20}:0, Arachidic acid	0.00–0.13	NR	NR
C_{21}:0, Heneicosylic acid	0.01–0.24	0.00–0.82	NR
C_{22}:0, Behenic acid	0.06	0.00–0.26	NR
C_{23}:0, Tricosylic acid	0.02	NR	NR
C_{24}:0, Lignoceric acid	0.02	NR	NR
Unsaturated fatty acid (UFA)			
Monounsaturated fatty acid (MUFA)	4.14–33.02	8.55–29.00	8.01–59.39
C_{16}:1n7, Palmitoleic acid	0.09–2.79	0.80–7.60	0.11–2.90
C_{18}:1n7, cis-Vaccenic acid	NR	0.12–0.43	NR
C_{18}:1n9, Oleic acid	1.54–30.23	5.66–22.70	3.55–57.63
C_{18}:1n9t, Elaidic acid	0.03	NR	NR
C_{20}:1n9, Gondoic acid	0.01–0.02	0.00–0.46	0.02–0.39
C_{22}:1n9, Erucic acid	0.01–0.52	NR	1.62
Polyunsaturated fatty acid (PUFA)	1.36–34.29	5.87–42.28	7.24–33.70
C_{18}:2n6, Linoleic acid	0.06–41.39	3.80–31.40	8.01–35.58
C_{18}:3n3, α-Linolenic acid	0.01–1.74	0.00–3.60	0.01–2.27
C_{18}:3n6, γ-Linolenic acid	0.01	NR	0.03–1.85
C_{18}:4n3, Stearidonic acid	NR	0.05–0.87	NR

Table 4. The fatty acid compositions of *Tenebrio molitor* compared to other protein sources. (continued)

Fatty acids (%DM[z])	*Acheta domesticus*[y] House cricket	*Hermetia illucens*[x] Black soldier fly larvae (BSF)	*T. molitor*[w] Yellow mealworm larvae (YML)
C_{20}:2n6, Dihomolinoleic acid	0.19	0.00–0.38	NR
C_{20}:3n3, Dihomo-α-linolenic acid	0.01	NR	NR
C_{20}:3n6, Dihomo-γ-linolenic acid	0.01	0.00–0.38	NR
C_{20}:4n6, Arachidonic acid	0.01	0.10–0.29	0.04–0.50
C_{20}:5n3, Timnodonic acid (EPA)	0.06–0.75	0.01–3.50	0.00–0.21
C_{22}:4n6, Adrenic acid	NR	0.10–0.29	0.13–0.46
C_{22}:5, Docosapentaenoic acid (DPA)	0.01–0.06	0.00–3.50	NR
C_{22}:6n3, Cervonic Acid (DHA)	NR	0.01–1.70	0.04–0.24
Omega-3 (n-3)	0.07–2.49	0.10–6.20	0.11–1.80
Omega-6 (n-6)	1.05–31.80	4.52–40.29	6.97–32.62
n-6/n-3	2.00–40.90	0.95–100.00	0.69–71.42

[z] DM: dry matter; NR: not reported.
[y] Data compiled from Pilco-Romero *et al*. (2023), Magara *et al*. (2021), Udomsil *et al*. (2019), and Nogales-Mérida *et al*. (2019).
[x] Data compiled from Vasilopoulos *et al*. (2024), Lu *et al*. (2022), Zulkifli *et al*. (2022), and Nogales-Mérida *et al*. (2019).
[w] Data compiled from Vasilopoulos *et al*. (2024), Shafique *et al*. (2021), Hong *et al*. (2020), and Nogales-Mérida *et al*. (2019).

Table 5. The mineral compositions of *Tenebrio molitor* compared to other protein sources.

Minerals (mg kg^{-1} DM[z])	*Acheta domesticus*[y] House cricket	*Hermetia illucens*[x] Black soldier fly larvae (BSF)	*T. molitor*[w] Yellow mealworm larvae (YML)	Soybean meal[v]	Fish meal[u]
Calcium (Ca)	275–3,150	1,200–45,150	168–5,000	1,600–4,660	55,500
Phosphorus (P)	2,250–10,389	1,000–11,500	700–14,290	5,640–7,660	31,300
Magnesium (Mg)	226–1,366	1,000–6,200	200–16,300	310–4,940	1,700
Potassium (K)	1,260–12,800	1,700–21,300	3,737–19,290	20,200–25,200	7,100
Sodium (Na)	950–8,633	600–15,600	404–3,644	60–1,090	7,100
Sulfur (S)	1	100–270	NR[z]	NR	NR
Manganese (Mn)[t]	9–44	134–166,000	3–19	30–71	36
Zinc (Zn)[t]	22–240	70–103,000	1–184	28–77	160
Copper (Cu)[t]	5–51	10–10,700	1–65	9–19	12
Iron (Fe)[t]	19–112	70–191,000	1–184	90–919	478
Selenium (Se)	1–6	NR	NR	NR	NR
Molybdenum (Mo)	0–635	NR	NR	NR	NR

[z] DM: dry matter; NR: not reported.
[y] Data compiled from Pilco-Romero *et al*. (2023), Ververis *et al*. (2022), Magara *et al*. (2021) and Udomsil *et al*. (2019).
[x] Data compiled from Vasilopoulos *et al*. (2024), Lu *et al*. (2022), and Zulkifli *et al*. (2022).
[w] Data compiled from Vasilopoulos *et al*. (2024), Syahrulawal *et al*. 2023, Shafique *et al*. (2021) and Hong *et al*. (2020).
[v] Data compiled from Ibáñez *et al*. (2020) and Hussein *et al*. (2017).
[u] Data compiled from Hussein *et al*. (2017).
[t] Belongs to heavy metals.

attacins, exhibit strong antimicrobial properties, capable of inhibiting the growth of various pathogens. Polysaccharides, such as chitin (4.3–8.9% DM) and chitosan, found in YML, have demonstrated antibacterial activity against bacteria such as *E. coli* and *S. enterica*. These bioactive compounds can enhance immune function, improve disease resistance, and support overall health, providing additional benefits in livestock nutrition and reducing the need for antibiotics (Elahi *et al*. 2022; Chen *et al*. 2023; Syahrulawal *et al*. 2023).

Case studies on the impact of YML on livestock growth performance

In recent years (2019–2024), several studies have explored the potential of using YML as alternative protein source in various farmed animals, including aquatic species such as red sea bream (*Pagrus major* (Temminck & Schlegel)) (Ido *et al*. 2019), largemouth bass (*Micropterus salmoides* (Lacepède)) (Chen *et al*. 2023), yellowtail (*Seriola quinqueradiata* Temminck & Schlegel) (Ido *et al*. 2024), giant river catfish (*Pangasianodon hypophthalmus* (Sauvage)) (Ardra *et al*. 2025), giant freshwater prawn (*Macrobrachium rosenbergii* (De Man)) (Chong *et al*. 2022), Chinese mitten crab (*Eriocheir sinensis* H. Milne Edwards) (Yao *et al*. 2024), as well as terrestrial farmed animals such as broiler chickens (Elahi *et al*. 2020), Japanese quail (*Coturnix japonica* Temminck & Schlegel) (Dalle Zotte *et al*. 2024), weaned pigs ((Yorkshire × Landrace) × Duroc) (Ao *et al*. 2020; Malla *et al*. 2024), steers (Carrasco & Drewery 2024), and rabbits (Volek *et al*. 2021). These studies investigated the feasibility of replacing fishmeal with live YML, whole-fat, or defatted YML powder, partially or completely, in animal feed. The evaluations primarily focused on growth performance, feed intake, digestibility, meat quality, and blood health indices (Table 6).

Balancing the nutritional composition of feeds is crucial for optimal growth and health in animals. Research has shown that maintaining the proper nutritional balance allows for the replacement of fishmeal with whole-fat or defatted

Table 6. Effects of *Tenebrio molitor* as animal feed on growth and physiological performance of farmed animals.

Farmed animals	YML[z] form	YML ratio in diets	Effects (compared to general diet)	References
Terrestrial animal				
Broiler chicks	Dried meal	4%	Increase in body weight and average daily gain during the starter phase.	Elahi *et al*. (2020)
	Fresh meal	10.48%	Decrease in animal hematological characteristics (decrease lysozyme concentration) and abdominal fat.	Elahi *et al*. (2020)
Coturnix japonica	Live	10%	Increase in feed intake but decrease in egg overall flavor.	Dalle Zotte *et al*. (2024)
(Yorkshire × Landrace) × Duroc	Dried meal	2%	Similar growth performance, apparent total tract digestibility of nutrients, blood profiles or fecal noxious gas emission.	Ao *et al*. (2020)
	Defatted dried meal	7.66%	Similar in growth performance and most gut health parameters, with an increase in the protection of the intestinal mucosa barrier function (reduce plasma diamine oxidase).	Malla *et al*. (2024)
Fattening rabbits	No mention	3%	Similar in the coefficients of total tract apparent digestibility and nitrogen output, despite a decrease in feed intake and body weight.	Volek *et al*. (2021)
Steers	Dried meal	Supplements (Isonitrogenous)	Stimulation in forage utilization (increase forage intake, total organic matter).	Carrasco & Drewery (2024)

Table 6. Effects of *Tenebrio molitor* as animal feed on growth and physiological performance of farmed animals. (continued)

Farmed animals	YML[z] form	YML ratio in diets	Effects (compared to general diet)	References
Aquatic animal				
Pagrus major	Defatted dried meal	65% (100% replacement of fish meal)	Increase in body weight gain rate.	Ido *et al*. (2019)
	Defatted dried meal	5–10%	Increase in survival rate after infection with *Edwardisella tarda*.	Ido *et al*. (2019)
Micropterus salmoides	Dried meal	5.28–10.56% (Isonitrogenous and isolipidic, 12–24% replacement of fish meal)	Increase in growth performance (final body weight, weight gain rate, specific growth rate), antioxidant capacity and immunity (enhance liver health).	Chen *et al*. (2023)
	Dried meal	15.58–21.23% (Isonitrogenous and isolipidic, 36–48% replacement of fish meal)	Decrease in growth performance and liver health.	Chen *et al*. (2023)
Seriola quinqueradiata	Full-fat dried meal	18.9% (Isonitrogenous and isolipidic)	Similar in growth performance.	Ido *et al*. (2024)
	Full-fat dried meal	10–20% (Isonitrogenous, not isolipidic)	Decrease in fork length and body weight.	Ido *et al*. (2024)
Pangasianodon hypophthalmus	Defatted dried meal	3.75–7.5% (Isonitrogenous and isolipidic, 7.07–14.14% replacement of fish meal)	Similar in the growth performance, whole body proximate composition, haematological and serum indices.	Ardra *et al*. (2025)
Eriocheir sinensis	Defatted dried meal	5.4–16.3% (Isonitrogenous and isolipidic, 25–75% replacement of fish meal)	Similar in the growth performance, intestinal health, serum immune, and antioxidant indexes.	Yao *et al*. (2024)
Macrobrachium rosenbergii	Live	Each treatment was fed GFP[z] twice a day (9 am and 4 pm) until visibly full	Although no significant differences were observed, the feed conversion ratio of the live mealworm feeding group (1.66) was higher than that of the other two groups (commercial feed (1.76); and a 50/50 formula of commercial feed and live mealworms (1.72)).	Chong *et al*. (2022)

[z] YML: yellow mealworm larvae; GFP: giant freshwater prawn.

dried YML powder, which not only promotes growth performance but also enhances animal health due to the bioactive compounds present in YML. However, when the replacement ratio of YML powder is too high or the feed's nutritional composition becomes unbalanced, the positive substitution effect may be significantly reduced. Ido *et al*. (2024) found that replacing 10% and 20% of the fishmeal with YML powder in feeds led to decreased body length and weight in yellowtail (*S. quinqueradiata*) as the replacement ratio increased. Chen *et al*. (2023) observed that when the replacement ratio of YML powder exceeded 36%, growth performance of largemouth bass (*M. salmoides*) significantly declined. Yao *et al*. (2024) reported that completely replacing fishmeal with defatted YML powder negatively impacted growth, serum immunity, and antioxidant indices in Chinese mitten crab (*E. sinensis*). A similar trend was observed in terrestrial farmed animals. Elahi *et al*. (2020) found that adding 10.48% fresh YML slurry to the diet reduced abdominal fat content in broiler chickens, while Volek *et al*. (2021) observed that adding 3% YML to the diet resulted in reduced feed intake and weight gain in rabbits.

The negative impact on growth and health may be attributed to several factors, including the excessive intake of chitin, a type of fiber found in YML. While many animals possess intestinal enzymes, such as chitinase, to break down chitin, excessive chitin intake can result in incomplete digestion. This can lead to the formation of indigestible complexes when chitin binds with amino acids, thereby interfering with digestion and absorption, which can stunt animal growth (Chen *et al*. 2023). Additionally, an imbalance in the fatty acid composition of the feed can adversely affect animal growth and health. For instance, a deficiency in omega-3 fatty acids can slow the growth of fish fry, while an excess of PUFAs may negatively impact the blood characteristics of broiler chickens (Elahi *et al*. 2020; Ido *et al*. 2024).

In conclusion, partial replacement of fishmeal with YML powder in animal feeds is a feasible approach. However, the replacement ratio must be carefully adjusted based on factors such as the species of farmed animals, feed freshness, and overall nutritional composition. Furthermore, the current market lacks large-scale production and a stable supply of YML powder, which presents challenges in accurately assessing its metabolizable energy and nutritional utilization efficiency. Therefore, further research is required to enhance the technology for large-scale application of YML powder in animal feeds, ensuring its feasibility and effectiveness in practical settings (Lee *et al*. 2024).

HIGH-VALUE APPLICATIONS OF YM ACROSS VARIOUS INDUSTRIES

With the UN-SDGs, large-scale insect farming is increasingly becoming a strategic industry in many countries, with the YML (*T. molitor*) and BSF (*H. illucens*) emerging as key representative species. YML has been widely incorporated into human food, pet food, and snacks (Ribeiro *et al*. 2018). Although insect-based proteins offer environmental, health, and nutritional benefits, their widespread acceptance remains limited. Therefore, exploring the development of economically valuable products derived from insects and their by-products such as medical materials, industrial raw materials, bioenergy, and aviation fuel, is considered a practical strategy to support the early-stage economics of insect farming. Owing to interdisciplinary advancements in industrial, biomedical, and food sciences, academic research, patents, and commercial applications related to such uses have steadily increased, demonstrating the feasibility of utilizing YML biomass as a high-value raw material. Given the substantial body of research and industrial investment already focused on YML as ingredients in human and pet food, these applications are excluded from present review. Instead, the following sections focus on diversified applications of high-quality products derived from YML reared on plant-based substrates, including agricultur-

al residues or by-products. These applications include commercialized products, prototypes under development, and innovations with promising future potential. Their viability depends on whether technological advancements can meet practical or economic thresholds. The objective of this section is to provide stakeholders including policymakers, circular agriculture practitioners, insect farmers, and potential industry adopters with a sustainable model that balances environmental and economic benefits. In doing so, insect farming may evolve into a green industry that contributes to food security, enhances human and animal nutrition, reduces carbon emissions, and minimizes waste, paving the way for long-term sustainability.

Animal husbandry and aquaculture

Feed additives are essential components in animal nutrition, formulated to promote health, enhance growth performance, and improve feed efficiency. The incorporation of insect meal into animal diets has been shown to prevent and treat diseases, stimulate growth, and enrich animal products with bioactive compounds. Studies have demonstrated that incorporating appropriate proportions of insect meal into the diets of farmed animals has minimal impact on the physicochemical or sensory properties of the resulting meat. However, notable alterations in the fatty acid profile have been observed (Bingqian *et al.* 2023). The effects of mealworm-based feed applications in both animal husbandry and aquaculture are summarized in Table 6.

Beyond its nutritional benefits, insect meal has shown promising potential as an alternative to antibiotics, whose overuse in animal production has raised global concerns due to the emergence of antibiotic-resistant bacteria and residual contamination in animal products, groundwater, soil, and feed. In response to these risks, EU implemented a ban in 2006 on the use of antibiotics as growth promoters in animal feed (Ghimpețeanu *et al.* 2022), that facilitates the search for viable substitutes. One promising substitute is YML meal, which has demonstrated beneficial effects on animal health and productivity. Islam & Yang (2017) reported that incorporating probiotics-fermented YML powder into broiler chick diets reduced the FCR, mortality rates, and concentrations of *E. coli* and *Salmonella* spp. in the cecum. Similarly, Ido *et al.* (2019) demonstrated that supplementing fish feed with 5–10% defatted YML powder significantly improved the survival rate of *P. major* infected with *Edwardsiella tarda* Ewing & McWhorter, a common fish pathogen. In parallel, Malla *et al.* (2024) showed that the inclusion of 7.66% defatted YML powder in weaned pig diets lowered plasma diamine oxidase levels, thereby enhancing mucosal barrier function and improving gut health. Collectively, these findings highlight the potential of YML meal as a functional feed ingredient that supports animal health, enhances performance, and reduces reliance on conventional antibiotics.

Biological control industry

YM can be mass-reared efficiently and provide stable yields at relatively low costs compared to natural hosts of many agriculturally important pests, making them ideal alternative hosts for the mass production of natural enemies used in biological control programs. Previous studies have identified their pupae as effective alternate hosts for parasitoids targeting a wide range of Lepidopteran pests, including species from the families Arctiidae, Geometridae, Oecophoridae, Pyralidae, Riodinidae, and Nymphalidae (Zanuncio *et al.* 2008; Favero *et al.* 2013; Li *et al.* 2019; Machado *et al.* 2023). Particularly, Li *et al.* (2019) reported that 1- to 2-day-old YM pupae are highly suitable as substitute hosts for *Chouioia cunea* Yang, a parasitoid used in the biological control of the fall webworm (*Hyphantria cunea* (Drury)). Rearing costs using YM pupae were significantly lower than those of the conventional host Chinese tussah (*Antheraea pernyi* Guérin-Méneville). Additionally, YML powder has also been used as a nutrient medium for culturing entomopathogenic nematodes

(Widiyaningrum *et al*. 2020). Given their role as alternate hosts for numerous natural enemies of crop pests, YM are frequently used in field experiments to monitor pest population dynamics and evaluate the effectiveness of natural enemies (Tschanz *et al*. 2007).

Plant protection industry

The mass-rearing of insects, including YM, generates substantial quantities of by-products such as frass (insect excreta) and exuviae (molted exoskeletons). For every 1 kg of YML reared, approximately 2–3 kg of by-products are generated (Zunzunegui *et al*. 2024). These by-products are rich in essential macronutrients nitrogen (N), P, and K as well as chitin, beneficial microorganisms, and trace minerals including sulfur (S), Ca, Mg, Mn, Fe, and molybdenum (Mo). Due to this nutrient-rich composition and compatibility with soil ecosystems, YML frass is increasingly recognized as a promising organic fertilizer and biostimulant with the potential to replace synthetic mineral fertilizers and chemical pesticides. Compared to traditional fertilizers, frass nutrients are more bioavailable to plants and can enhance the abundance of plant growth-promoting rhizobacteria (PGPR), indirectly improving crop growth and yield. Moreover, frass application can boost plant tolerance to abiotic stresses such as salinity, drought, and flooding. The chitin present in frass has also been shown to trigger plant defense responses, thereby increasing resistance to pests and diseases (Poveda 2021; Barragán-Fonseca *et al*. 2022; Zunzunegui *et al*. 2024). Zunzunegui *et al*. (2024) reviewed studies published between 2019 to 2023, demonstrating the positive effect of YML frass on crop growth and resilience to environmental stress. The present work extends this review by incorporating research published through the end of 2024, as summarized in Table 7 and Table 8. As with conventional fertilizers, appropriate application methods and dosages of frass must be carefully managed based on crop species and developmental stages. Overapplication may lead to excessive soil salinity, fertilizer burn, compaction, or waterlogging, all of which can inhibit nutrient uptake and negatively impact plant growth (Zhang *et al*. 2012; Liu *et al*. 2019; Zunzunegui *et al*. 2024). The use of frass as a plant fertilizer is gaining international attention. Several commercial products have emerged, such as a pelletized frass fertilizer (5 mm granules) produced by the German company Organifer (https://organifer.com/en/), which complies with EU organic farming regulations. This product offers a nutrient composition of NPK (nitrogen, phosphorus, potassium) (3.5-3.5-2.5), along with Ca (2%), and Mg (0.7%), can be broadcast or mixed into the soil, and has low carbon emissions compared to synthetic fertilizers. Another example is a powdered frass product developed by the U.S.-based KIS Organics (https://www.kisorganics.com/), which is suitable for direct soil application, foliar spray, or use in hydroponic systems. Nevertheless, the effectiveness of frass-based fertilizers depends on multiple factors such as crop type, soil characteristics, climate conditions, and nutrient availability. Further research is necessary to optimize application protocols for scaling up its use in commercial agriculture. Such advancements could reduce dependence on synthetic fertilizers and pesticides, thereby promoting more sustainable agricultural practices. In addition to its value as fertilizer, frass also serves as a promising feedstock for biochar production. When pyrolyzed, insect frass yields high-efficiency biochar that not only enhances carbon sequestration but also exhibits strong adsorption capacities for heavy metals, making it effective for soil remediation. Compared to biochar derived from crop residues, insect-based biochar shows superior performance in adsorbing lead (Pb), Cd, chromium (Cr), Cu, and Zn (Yang *et al*. 2019; Moruzzo *et al*. 2021). These diverse applications significantly enhance the value of insect farming by-products and support innovations in circular agriculture and ecologically friendly technologies. In addition to insect frass as a fertilizer, Urrutia *et al*. (2023) demonstrated that bio-oil derived from pyrolyzed YML frass could serve as a novel biopesticide, effectively controlling

Table 7. Researches on the use of *Tenebrio molitor* frass to enhance plant growth.

Plant	Effects	References
Amaranthaceae		
Beta vulgaris L.	Increase biomass of dry plant.	Przemieniecki *et al.* (2024)
Araceae		
Philodendron pedatum (Hook.) Kunth	Increase growth and leaf greenness.	Baranimotlagh *et al.* (2024)
Araliaceae		
Panax ginseng CA Mey	Increase seed germination, plant height, leaf length, leaf width, root length, and root weight.	Kim *et al.* (2023)
Asteraceae		
Helianthus annuus L.	Increase biomass of roots, stem, flower head, whole plant and nutrition (N, P, K).[z]	Foscari *et al.* (2024)
Helminthotheca echioides (L.) Holub	Increase edible biomass (leaves number, rosette diameter, root fresh and dry weight), nutrition (P, K).[z]	Karkanis *et al.* (2025)
Sonchus oleraceus L.	Increase edible biomass (leaves number, rosette diameter, root fresh and dry weight), nutrition (P, K, Fe, Zn).[z]	Karkanis *et al.* (2025)
Brassicaceae		
Brassica oleracea L.	Increase fresh shoot weight.	van de Zande *et al.* (2024)
Brassica rapa L.	Increase leaf area.	Chia *et al.* (2024)
Strophariaceae		
Agrocybe chaxingu NL Huang	Increase edible biomass (flush fruitbody yield).	Zeng *et al.* (2017)

[z] N: nitrogen; P: phosphorus; K: potassium; Fe: iron; Zn: zinc.

Table 8. Researches on the use of *Tenebrio molitor* frass that enhances tolerance against abiotic or biotic stresses.

Plant	Effects	Abiotic/biotic stresses	References
Brassicaceae			
Arabidopsis thaliana (L.) Heynh.	Increase tolerance to biotic stress (activated systemic defenses).	*Botrytis cinerea*	Blakstad *et al*. (2023)
Fabaceae			
Phaseolus vulgaris L.	Increase tolerance to abiotic stress	Salinity, drought, flooding	Poveda *et al*. (2019)
	Increase tolerance to biotic stress (decrease disease index and incidence).	*Rhizoctonia solani*	Moarrefzadeh *et al*. (2023)

important stored-product pests such as Indian meal moth (*Plodia interpunctella* (Hübner)) and red flour beetle (*Tribolium castaneum* (Herbst)), as well as sanitary pest such as common house mosquito (*Culex pipiens* Linnaeus).

Educational materials in life sciences

To reduce the cost of larval feed, mealworm farming operations worldwide frequently use plant-based residues and by-products from the agriculture and food industries as feeding substrates. Beyond their dietary flexibility, YML have also demonstrated the ability to ingest and degrade polystyrene foam, a widely used synthetic plastic that poses significant environmental challenges. This capacity for plastic biodegradation has garnered increasing attention to YML as potential agents for plastic waste management and environmental sustainability (Siddiqui *et al*. 2024). Because of these distinctive traits, YML are increasingly used as model organisms in natural science education, environmental research, and circular agriculture studies.

Bioenergy industry

Fossil fuels are unsustainable resources, and the ongoing global energy transition necessitates the development of alternative energy solutions to reduce reliance on them. Insect oil, a form of animal fat and a key by-product of insect farming, has emerged as a promising feedstock for biofuel production. Studies have shown that insects reared on agricultural, industrial, or municipal organic by-products can accumulate substantial amounts of high-quality lipids, particularly fatty acids in the C_{16}–C_{18} range. These lipids can be converted into biodiesel using various technologies. For instance, transesterification reactions catalyzed by sodium phosphate have been used to efficiently convert YM oil into biodiesel that meets international fuel quality standards, including ASTM D6751 and EN 14214 (Siow *et al*. 2024). Consequently, insect oil is considered a novel and sustainable raw material for future biofuel production (Manzano-Agugliaro *et al*. 2012; Surendra *et al*. 2016; Baruah *et al*. 2023; Koyunoğlu 2024; Siow *et al*. 2024). Furthermore, Lee *et al*. (2022) demonstrated that YM fed with wheat bran can rapidly convert dietary carbohydrates into lipids during their larval stage. Notably, YM has a shorter growth cycle compared to many conventional oil-producing organisms, reinforcing their potential as a rapid and efficient biomass source for biofuel production. Compared to fossil fuels or traditional biofuels derived from energy crops (such as ethanol from corn or sugarcane), insect-derived oils offer superior energy efficiency and a lower carbon footprint (Koyunoğlu 2024). These characteristics make them attractive candidates for conversion into aviation-grade fuels, particularly as the aviation industry seeks to expand its use of sustainable aviation fuels (SAFs). The successful industrialization of biodiesel production from insect oils depends on the careful selection of feedstock materials, as the organic substrates used to rear insects influence the fatty acid composition of species such as the BSF and the YM. Optimizing substrate selection within circular agriculture systems is therefore essential to maximize both the quality and applicability

of insect-derived oils. In addition to biodiesel, YM farming can also contribute to bioenergy production via anaerobic digestion. The residual feed and frass mixtures generated during large-scale mealworm production can be fermented to produce methane-rich biogas (Bulak *et al*. 2020). This represents a valuable energy by-product that further enhances the sustainability and resource efficiency of insect-based bioconversion systems.

Industrial ingredients

Chitin and chitosan derived from YML exhibit potential for industrial applications. Compared to crustacean-derived chitin, YML-derived chitin is softer and demonstrates superior anti-inflammatory properties, making it a uniquely advantageous raw material for the development of novel industrial products (Son *et al*. 2021). Insect-derived chitin has also been considered as an adsorbent material for the removal of dyes in the textile, leather, and paper industries. Jóźwiak *et al*. (2023) demonstrated that YML-derived chitin effectively adsorbs anionic dyes. Furthermore, Nettey-Oppong *et al*. (2025) reported that YML-derived chitosan shows promise as a sustainable sensing material for use in flexible humidity sensors, indicating potential applications in environmental monitoring, smart agriculture, and industrial process control.

Cosmetic industry

Insect-derived oils, proteins, and chitinous compounds (such as chitin and chitosan) are increasingly being explored as high-value by-products for cosmetic applications (Albushabaa & Haider 2022; Bingqian *et al*. 2023). Lipids extracted from YM contain a significant proportion of UFAs, particularly linoleic and linolenic acids. These essential fatty acids help reduce water loss through epidermis and act as lipid barriers that support skin regeneration, making them promising alternatives to plant-derived oils in cosmetic formulations (Verheyen *et al*. 2023). In addition, YML proteins are rich in amino acids, while their chitin exhibits antioxidant and antimicrobial properties (Kim *et al*. 2018; Nafary *et al*. 2023). Due to this unique biochemical composition and functional profile, YML-derived ingredients hold considerable potential as substitutes or novel components in a wide array of cosmetic products. This represents a promising economic opportunity for large-scale YM farmers, particularly those in the early stages of industrial development. By tailoring rearing and processing methods to meet specific product requirements, producers can apply suitable post-harvest treatments such as cleaning, freezing, or drying that are both cost-effective and compliant with cosmetic regulations. Target bioactive compounds can then be selectively extracted to be used either as functional ingredients (such as oils, waxes, emulsifiers) or as active ingredients (such as antioxidants, antimicrobials) across various cosmetic applications. These include skin-protective products such as hand creams, skincare creams, lip balms, and conditioner as well as cleansing formulations (such as soaps and body washes) and exfoliating treatments (such as face mask and exfoliant). However, the successful integration of YML-based ingredients into the cosmetic industry is contingent upon overcoming several challenges, including the development of safe and efficient biorefining techniques, strict quality control during production, and full compliance with cosmetic safety regulations across different countries and economic regions.

Food packaging industry

Conventional plastic packaging, primarily derived from petroleum-based sources, has greatly enhanced the convenience of modern life. However, both its production and disposal particularly via incineration are significant contributors to carbon emissions. This has spurred increasing interest in developing environmentally friendly alternatives to petroleum-based materials, thereby accelerating the advancement of the green packaging industry. Weng *et al*. (2023) reviewed the application of insect-derived proteins and chitosan as raw materials for food packaging films, revealing that chitosan-based

films generally exhibit superior tensile strength compared to protein-based films. Notably, Mwita *et al*. (2024) demonstrated that chitosan films derived from YML biomass effectively extended the shelf life of bananas, highlighting their practical potential in postharvest preservation. In terms of antimicrobial functionality, protein-based films often require the incorporation of external antimicrobial agents, whereas chitosan inherently exhibits antimicrobial properties. Liu *et al*. (2024) further enhanced the physicochemical properties of chitosan films by incorporating YML proteins to improve their antioxidant and antimicrobial efficacy through the addition of propolis ethanol extract. These biodegradable and bioactive packaging materials offer promising solutions for sustainable food packaging, particularly in extending the shelf life of fresh produce and reducing reliance on petroleum-based plastics.

Biomedical materials and prospective medical research

Although chitin and its deacetylated derivative, chitosan, have been widely studied in biomedical applications, the exploration of insect-derived sources remains in its early stages. In particular, chitin and chitosan extracted from YML hold promise as sustainable and functional alternatives to conventional crustacean-derived materials (Mei *et al*. 2024), with potential applications in wound healing, tissue engineering, drug delivery, and antimicrobial therapies.

Beyond material applications, YML themselves have recently gained increasing recognition as alternative invertebrate models in medical research. Studies have demonstrated their suitability for investigating microbial pathogenesis and assessing drug toxicity, providing a cost-effective and ethically preferable substitute for conventional mammalian models such as rodents. For instance, de Souza *et al*. (2015) first validated YML as hosts for two major human fungal pathogens, *Cryptococcus neoformans* (San Felice) Vuill. and *Candida albicans* (CP Robin) Berkhout, to clarify the mechanisms of microbial pathogenesis. Subsequent studies confirmed their relevance for evaluating the virulence of *Sporothrix* species (Lozoya-Pérez *et al*. 2021), while more recent work by Brai *et al*. (2023) established a novel YML-based platform for preclinical testing of antiviral and antimicrobial agents. Collectively, these findings underscore the growing potential of YML and their derivatives as versatile resources for biomedical innovation.

CONCLUSION AND FUTURE PERSPECTIVES

With the increasing global demand for food and the mounting pressure of waste management, identifying sustainable protein sources and implementing effective resource recycling strategies have become critical priorities. Due to their high nutritional value, low environmental footprint, and capacity to biodegrade agricultural residues, YM have emerged as one of the key decomposers of plant-based waste within circular agriculture systems. They serve as a significant source of animal-derived protein for livestock feed, pet food, and treats. In Europe and the Americas, numerous companies are actively developing YML-based products for human consumption. Over the past decade, the utilization of YML as raw materials for high-value products has become a strategic focus in several national development plans. Current research indicates that YML can thrive on various plant-based substrates, such as cereal by-products and fruit and vegetable waste. However, their growth performance depends highly on the protein-to-carbohydrate ratio of the substrate, which must be carefully adjusted to optimize rearing efficiency. Concurrently, ensuring product safety requires rigorous monitoring for contaminants such as pesticide residues, heavy metals, microplastics, pathogenic microorganisms, and mycotoxins, particularly for products intended for animal feed or human consumption.

YML meal, characterized by a favorable amino acid and fatty acid composition, has been demonstrated to partially replace fishmeal and

soybean meal in both livestock and aquaculture diets. Its inclusion can enhance growth performance, immunity, and feed conversion efficiency in livestock and aquatic species. However, excessive substitution levels or imbalanced nutrient profiles may negatively affect animal health, necessitating species-specific and nutritionally precise feed formulations. Furthermore, the by-products of YML farming such as frass and exuviae are rich in N, P, K, and chitin. These materials can be used in organic fertilizers, soil amendments, and stimulants, contributing to improved crop growth and disease resistance, and further expanding their value in circular agricultural systems.

When YML is reared on non-plant substrates (such as kitchen waste containing animal residues or animal by-products), safety concerns generally preclude their use in food or feed applications. However, the extracted lipids from such systems remain suitable for the bioenergy sector, including biodiesel and SAF production. In parallel, chitin derived from YM has shown promise in environmental applications, such as biosorption of heavy metals and water purification- offering added economic value beyond the food chain.

In summary, the sustainable development of the YM farming industry relies on clearly defining the types of end products and their intended applications, which in turn determines the appropriate categories of feed substrates. Such classification is essential to achieve sufficient economic scale to support industrial growth. Production systems should be designed to meet labor- and energy-saving requirements, ideally through semi-automated or fully automated farming technologies. Equally important, government authorities must establish timely and adaptive regulatory frameworks and policy instruments to guide the industry. From a technical perspective, nutritional regulation of YMs differs across breeding stages and larval growth phases, requiring tailored feed formulations to meet specific farming objectives. Risk management during rearing is also critical, including the monitoring of feedstock contamination and the control of insect-borne pathogens. Safety assessments should be conducted both on the input feed materials and on the harvested larvae to eliminate the risk of harmful residues. Furthermore, standardized farming and production protocols need to be developed so that different producers can deliver consistent, safe, and regulation-compliant products. Only by ensuring product reliability and public trust can YM farming achieve sustainable, large-scale industrialization.

ACKNOWLEDGMENTS

We sincerely appreciate Hao-Wen Duan, Cheng-Lung Chu, Zong-Tze Hong, and Jyue-Min Jhang for assisting with collecting information on the nutrition composition of insects. We also extend our gratitude to Mu-Xuan Chen for contribution to the preliminary design of illustrations. This work was financially supported by the research programs 114AS-14.1.3-CI-01 and 114AS-2.5.2-CI-01 from the Ministry of Agriculture.

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以植物基質飼養麵包蟲的多元應用：循環農業、動物飼料及其他高值化產品

謝孟祐 [1,*] 馮文斌 [1,*] 許北辰 [2] 董耀仁 [2] 李雅琳 [3] 石憲宗 [4,**]

摘要

謝孟祐、馮文斌、許北辰、董耀仁、李雅琳、石憲宗。2025。以植物基質飼養麵包蟲的多元應用：循環農業、動物飼料及其他高值化產品。台灣農業研究 74(4):415–447。

隨著全球人口持續增長，糧食供應壓力與廢棄物管理挑戰日益嚴峻，昆蟲已被視為解決此問題的重要關鍵之一。昆蟲作為有機廢棄物的生物轉化者，能將農業副產品與食品加工廢棄物等難以應用的資源轉化為有用的資源，不僅實現資源的循環再利用同時降低碳足跡，亦可有效取代傳統養殖動物飼料的蛋白質來源 (如大豆與魚粉)，從而促進農業與畜牧業的持續性發展。昆蟲可作為動物養殖飼料的原料，昆蟲含高量蛋白質與脂肪，能有效替代傳統動物飼料的魚粉等動物源蛋白質成分，為畜牧業與水產養殖之永續經營提供良好的替代方案。由於昆蟲養殖具有資源投入低、生長週期短以及生產空間需求小等優勢，使其成為一種高效且環境友善的資源利用模式，進一步推動農業朝永續發展轉型，並促進循環經濟的實現。除蟲體生物質之外，養殖過程中產生的蟲糞也是重要的副產物，其富含氮、磷、幾丁質及有益微生物，具有開發為有機肥料促進植物生長的作用，更進一步強化了麵包蟲養殖於永續循環系統中的潛力。本研究以麵包蟲 (*Tenebrio molitor*) 為例，探討其分解植物性廢棄物質的可行性與優勢，並分析飼養過程中的關鍵考量，同時評估麵包蟲幼蟲作為動物養殖飼料原料的潛力 (包含營養價值與應用案例)，以及蟲體與副產品開發成高經濟價值終端產品的應用前景。本文旨在為資源昆蟲於全球糧食供應與環境永續發展的研究，以及未來的開發方向提供完整的參考資料。相較於傳統作物與畜禽養殖，昆蟲養殖產業展現出更高的資源利用效率與更低的環境衝擊，是符合聯合國永續發展目標 (SDGs) 的綠色產業之一。

關鍵詞：麵包蟲、植物基質、循環農業、養殖動物飼料、高值化應用。

投稿日期：2025 年 7 月 8 日；接受日期：2025 年 10 月 7 日。
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台灣農業研究 (J. Taiwan Agric. Res.) 74(4):449–458 (2025)
https://doi.org/10.6156/JTAR.202512_74(4).0005

Research Article

Etiology and Fungicide Screening of Avocado Leaf Spot Disease Caused by *Pseudoplagiostoma perseae*

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Abstract

Wu, C. J, S. L. Hsu, and S. Y. Lai. 2025. Etiology and fungicide screening of avocado leaf spot disease caused by *Pseudoplagiostoma perseae*. J. Taiwan Agric. Res. 74(4):449–458.

Avocado leaf spot disease, caused by *Pseudoplagiostoma perseae*, is increasingly observed in avocado orchards across Taiwan. Despite its prevalence, limited information is available on disease dynamics and effective control measures. Field surveys conducted in this study revealed that disease severity peaks during the fall and winter, with disease decline occurring in spring. Laboratory assays showed that the optimal temperature for mycelial growth is 25℃, while the optimal temperature for spore germination ranges from 20℃ to 30℃. Pathogenicity tests confirmed that *P. perseae* induces characteristic pinpoint-like spots with yellow halos on the leaves of avocado cultivars ‘Hass’, ‘Pinkerton’, and ‘Tainung No.1 Tasty Red’ within 2 wk post-inoculation. Among the fungicides tested at 1 mg a.i. L^{-1}, pyraclostrobin completely inhibited mycelial growth. In addition, azoxystrobin, pyraclostrobin, trifloxystrobin, azoxystrobin + difenoconazole, fluopyram + trifloxystrobin, thiabendazole, and fluazinam completely suppressed spore germination at 1 mg a.i. L^{-1}. These findings contribute to a better understanding of the epidemiology of *P. perseae* and provide guidance for the development of effective disease management strategies in avocado cultivation.

Key words: Avocado, Leaf spot, Pathogenicity, Temperature, Fungicide.

INTRODUCTION

Avocado (*Persea americana* Miller) is one of the world’s most economically important fruit crops, with global demand continuing to rise. In 2023, avocado fruit exports from Mexico increased by 27% compared to the previous year (FAO 2024). In Taiwan, the avocado cultivation area reached 2,062 ha in 2023, representing a more than 3.5-fold increase compared to 2013 (https://agrstat.moa.gov.tw/sdweb/public/inquiry/InquireAdvance.aspx). A diverse array of cultivars is grown in Taiwanese orchards, including ‘Chang-an’, ‘Hung Shin Yuan’, ‘Hall’, ‘Choquette’, ‘Pinkerton’, ‘Tainung No. 1 Tasty Red’, ‘Tainung No. 2 Green Gold’, and ‘CAES-1’ to ‘CAES-4’.

Avocado leaves are susceptible to a range of fungal pathogens, with at least 6 species reported to cause leaf lesions or spots. *Sphaceloma perseae* causes black lesions that are localized to the midrib and main veins (Jenkins 1925, 1934). *Cercospora purpurea* produces light- to purplish-brown leaf spots often encircled by narrow yellow halos (Pohronezny *et al*. 1994). *Colletotrichum* spp., although mainly associated with postharvest fruit rot, can also infect leaves, initiating as yellow discolorations that progress

Received: May 13, 2025; Accepted: July 30, 2025.
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to brown necrotic lesions, especially at leaf tips (Dreistadt 2008; Giblins *et al*. 2010). Both *Bipolaris setariae* and *Corynespora cassiicola* cause pinpoint-like spots with yellow halos during early infection stages (Qiu *et al*. 2020; Wang *et al*. 2024).

Recently, *Pseudoplagiostoma perseae*, the causal agent of avocado leaf spot disease was identified in Taiwan (Wu *et al*. 2024). The initial symptoms present pinpoint-like spots with yellow halos, which develop into brown lesions with purple-brown margins and dark brown centers. This species is one of two *Pseudoplagiostoma* spp. reported to affect economic fruit trees. The other, *P. mangiferae*, was originally isolated from mango leaves exhibiting blight symptoms (Phookamsak *et al*. 2019), and its pathogenicity has been experimentally confirmed (Zhou *et al*. 2022). Cross-inoculation tests demonstrated that *P. perseae* and *P. mangiferae* exhibit host specificity, with infections restricted to their respective original hosts (Haituk *et al*. 2024; Wu *et al*. 2024).

Although several fungicides- mancozeb, carbendazim, prochloraz, and trifloxystrobin- effectively inhibit mycelial growth of both *Pseudoplagiostoma* species (Haituk *et al*. 2024), comprehensive knowledge regarding the epidemiology and management of *P. perseae* remains limited. This study aims to elucidate the field occurrence of *P. perseae*, determine the optimal temperature conditions for mycelial growth and spore germination, and evaluate the efficacy of selected fungicides for disease control.

MATERIALS AND METHODS

Field survey

The field surveys were conducted in Shanshang, Tainan (23°05'45.4"N, 120°21'42.1"E) and Chiayi City (23°29'6.79"N, 120°28'27.23"E). In Shanshang, Tainan, the survey period spanned from August 2020 to March 2021, whereas in Chiayi City, observations were carried out from August 2020 to August 2021. For each avocado cultivar surveyed, a minimum of four trees were examined. On each tree, 20 shoots were randomly selected, and 5 leaves per shoot were assessed for disease severities. Disease severity was categorized as follows: 0- no symptoms; 1- disease area covering 1 to 5% of the leaf area; 2- disease area covering 5 to 25%; 3- disease area covering 26 to 50%; 4- disease area exceeding 50%. Disease severity was calculated using the following formula: [Σ(leaf severity rating)/(total number of leaves × 4)] × 100%, where 4 represents the maximum disease severity score per leaf.

Effect of temperature on mycelial growth

P. perseae isolates V-019 and V-027 were used in this assay. The isolates were incubated at 25℃ for 2 wk. Fungal discs (0.5 cm in diameter) were cut from the colonies and placed on potato dextrose agar (PDA; Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The plates were incubated in darkness at different temperatures, ranging from 10℃ to 40℃. Each temperature treatment included six replicates. Colony diameters were measured 21 d after incubation. The experiment was repeated twice.

Effect of temperature on spore germination

For sporulation, *P. perseae* isolates V-019 and V-027 were incubated in darkness at 25℃ for 21 d. A spore suspension was prepared using 0.02% Tween 20. The concentration of the spore suspension was adjusted to 5 spores μL^{-1}, and 100 μL of the suspension was evenly spread on a PDA plate. The plates were incubated at different temperatures, ranging from 10℃ to 40℃. Twenty-four hours after incubation, one hundred spores from each plate were examined under a microscope. A spore was considered germinated if its germ tube was at least 2.5 times longer than the spore itself. Each temperature treatment included six replicates, and the experiment was repeated twice.

Fungicide inhibition test on mycelial growth

The fungicides used in this assay included the following: 23.0% azoxystrobin suspension concentrate (SC; Syngenta Taiwan Ltd., Taipei,

Taiwan), 62.5% cyprodinil + fludioxonil water dispersible granules (WG; Syngenta Taiwan Ltd., Taipei, Taiwan), 70% thiophanate-methyl wettable powder (WP; Lih-Nung Chemical Co., Ltd., Yunlin, Taiwan), 23.6% pyraclostrobin emulsifiable concentrate (EC; Wonderful Agro Co., Ltd., Taichung, Taiwan), 40% iminoctadine tris WP (Sumitomo Cooperation Taiwan Ltd., Taipei, Taiwan), 25.9% tebuconazole emulsion (EW; oil-in-water; Bayer Taiwan Ltd., Taipei, Taiwan), 80.0% metiram WG (BASF Taiwan Ltd., Taipei, Taiwan), 50.0% trifloxystrobin WG (Bayer Taiwan Ltd., Taipei, Taiwan), 32.5% azoxystrobin + difenoconazole SC (Syngenta Taiwan Ltd., Taipei, Taiwan), 50.0% fluopyram + trifloxystrobin SC (Bayer Taiwan Ltd., Taipei, Taiwan), 40.0% thiabendazole WP (Fulon Chemical Industrial Co., Ltd., Taipei, Taiwan), and 39.5% fluazinam SC (ISK Taiwan Ltd., Taipei, Taiwan). Fungicides were added into PDA at concentrations of 1, 10, or 100 mg a.i. L^{-1}. PDA without fungicides served as the control treatment. *P. perseae* isolates V-019 and V-027 were cultured on PDA at 25℃ for 2 wk. Mycelial plugs (0.5 cm in diameter) were excised from the margins of actively growing colonies and placed onto PDA plates amended with the respective fungicides. After 2 wk of incubation at 25℃ in darkness, colony diameters were measured. Each treatment consisted of 6 replicates, and the entire experiment was repeated twice. The inhibition rate was calculated using the following formula: $[(C - T)/C] \times 100\%$, where C is the average colony diameter in the control and T is that in the treatment.

Fungicide inhibition test on spore germination

The preparation method for PDA plates containing fungicides was the same as that of the fungicide test on mycelial growth. Spores are suspended with 0.02% Tween 20 and adjusted to a concentration of 5 spores μL^{-1}. One hundred μL of spore suspension was spread evenly on a PDA plate containing fungicides. The plates were incubated at 25℃ in darkness for 24 h. The spore germination was inspected under a microscope. Each treatment included six replicates, and the experiment was repeated twice. The spore germination rate was calculated using the following formula: $[(Gc - Gt)/Gc] \times 100\%$, where Gc is the germination rate in the control and Gt is that in the treatment.

Pathogenicity assay

One-year-old seedlings of the cultivars 'Hass', 'Pinkerton', and 'Tainung No.1 Tasty Red' were used in the assay. For each cultivar, three plants were inoculated, with two young leaves per plant selected for inoculation. For sporulation, *P. perseae* isolate V-027 was incubated on PDA for 21 d at 25℃ in darkness. The inoculum was a spore suspension at a concentration of 2×10^4 spores mL^{-1}. The spore suspension was spread on both the upper and lower surfaces of young leaves until runoff. The negative control was treated with sterilized water. The inoculated leaves were sealed in zipper bags for 2 d. Symptoms were observed 14 d after inoculation. To fulfill Koch's postulates, *P. perseae* was re-isolated from symptomatic leaf spots and identified based on its morphological traits and amplified *TUB* sequences. The experiment was repeated twice.

RESULTS

Field investigation

The investigation started in August 2020 in 2 orchards (Fig. 1). In the orchard in Chiayi City (Fig. 1A), the cultivars 'Tainung No. 1 Tasty Red', 'CAES-4', and 'Pinkerton' were examined. In October 2020, the disease severity exceeded 20% on 'CAES-4' and 'Pinkerton'. From March to June 2021, the disease severity was less than 10% in all three cultivars, and the disease severity increased rapidly on 'Pinkerton' and 'CAES-4' in July 2021. The relative humidity, precipitation, and temperature of Chiayi City are shown in Fig. 1C. In the orchard of Shanshang, Tainan (Fig. 1B), the cultivars 'CAES-2', 'Tainung No. 1 Tasty Red', and 'Hung Shin Yuan' were examined.

In November 2020, the disease severity on 'Tainung No. 1 Tasty Red', and 'Hung Shin Yuan' was 25% and 30%, respectively. In August 2020 and March 2021, the disease severity of the three cultivars was less than 10%. The relative humidity, precipitation, and temperature of Shanshang, Tainan are shown in Fig. 1D.

The temperature effect on mycelial growth

The optimal temperature of mycelial growth for *P. perseae* was 25℃, and the growth rate was 3.8 to 4.0 mm d^{-1} (Fig. 2). The mycelial growth ceased at 35℃. While at 10℃, the mycelial growth was less than 1.0 mm d^{-1}.

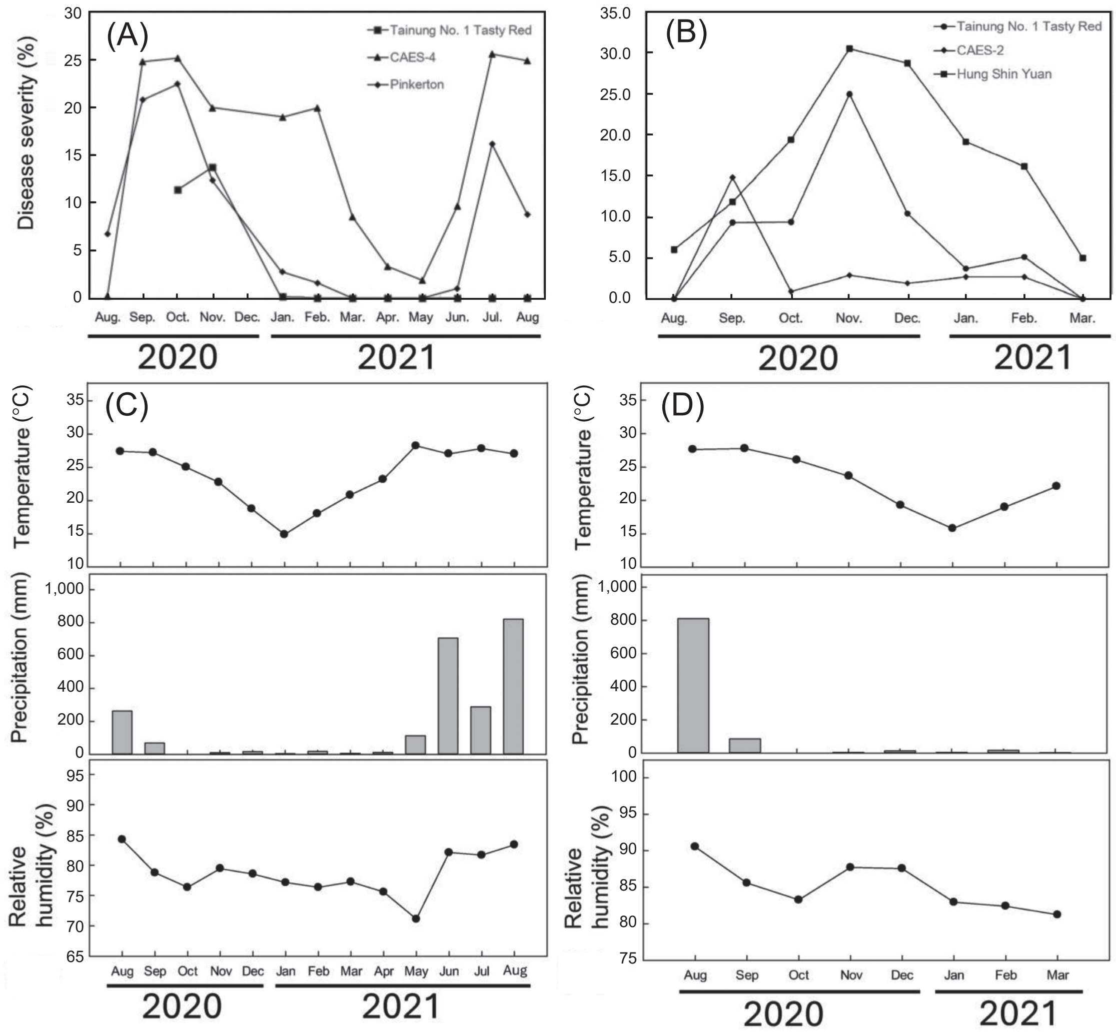

Fig. 1. Field survey of avocado leaf spot disease with weather data: (A) From August 2020 to August 2021 in Chiayi City; (B) from August 2020 to March 2021 in Shanshang, Tainan. The monthly average of relative humidity, precipitation, and monthly average of air temperature of (C) Chiayi city and (D) Shanshang, Tainan. Disease severity = [Sum of disease severity of investigated leaves/(total number of investigated leaves × 4)] × 100%.

The temperature effect on spore germination

The optimal temperature range for spore germination of *P. perseae* is 20 to 30℃ (Fig. 3). The germination rates of isolates V-019 and V-027 at 25℃ were 65.8% and 67.7%, respectively. At 35℃, the germination rate dropped to below 30%. The spore germination of *P. perseae* ceased at 10℃ and 40℃.

Pathogenicity test

Needle-like spots appeared on avocado cultivars 'Hass', 'Pinkerton', and 'Tainung No. 1 Tasty Red' 2 wk after inoculation with *P. perseae* (Fig. 4). On 'Hass' and 'Pinkerton' (Fig. 4A and 4B), the needle-like spots were surrounded by a distinct yellow halo. In contrast, the spots on 'Tainung No. 1 Tasty Red' were surrounded by a fuzzy halo or lacked a halo (Fig. 4C). The negative control, inoculated with sterilized water, showed no symptoms (Fig. 4D). To fulfill Koch's postulates, the symptomatic leaves were surface-disinfected, and fungi were re-isolated from the lesions. *P. perseae* was re-isolated from all inoculated leaves and identified based on colony morphology and β-tubulin (*TUB*) sequence.

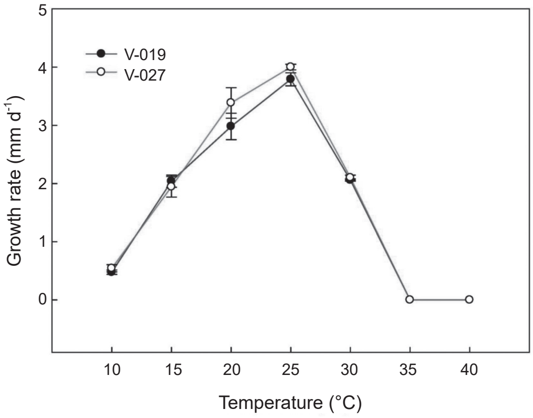

Fig. 2. Effect of temperature on mycelial growth of *Pseudoplagiostoma perseae* (isolates V-019, V-027).

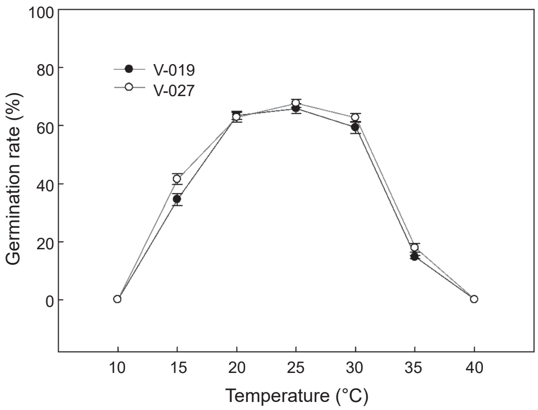

Fig. 3. Effect of temperature on spore germination of *Pseudoplagiostoma perseae* (isolates V-019, V-027).

Inhibitory effects of fungicides on mycelial growth

At a concentration of 1 mg a.i. μL^{-1}, pyraclostrobin effectively inhibited the mycelial growth of *P. perseae*, with an inhibition rate of 100% (Table 1). At 10 mg a.i. μL^{-1}, the inhibition rate of six fungicides, namely thiophanate-methyl, pyraclostrobin, iminoctadine-tris, tebuconazole, and azoxystrobin + difenoconazole, and thiabendazole, was 100%. At 100 mg a.i. μL^{-1}, all tested fungicides inhibited more than 80% of *P. perseae* mycelial growth, except for azoxytrobin, trifloxystrobin, and fluopyram + trifloxystrobin.

Inhibitory effects of fungicides on spore germination

At the concentration of 1 mg a.i. μL^{-1}, azoxystrobin, pyraclostrobin, trifloxystrobin, azoxystrobin + difenoconazole, fluopyram + trifloxystrobin, thiabendazole, and fluazinam effectively inhibited the spore germination of *P. perseae* (Table 2). At the concentration of 100 mg a.i. μL^{-1}, all tested fungicides completely inhibited spore germination, except for iminoctadine-tris. However, in iminoctadine-tris treatment, the spore germination rate was only 3.3% to 5.7%. The spore germination rate in the negative control (without fungicide application) ranged from 75.8% to 83.8%.

DISCUSSION

The avocado leaf spot disease caused by *P. perseae* has only been reported in Taiwan and

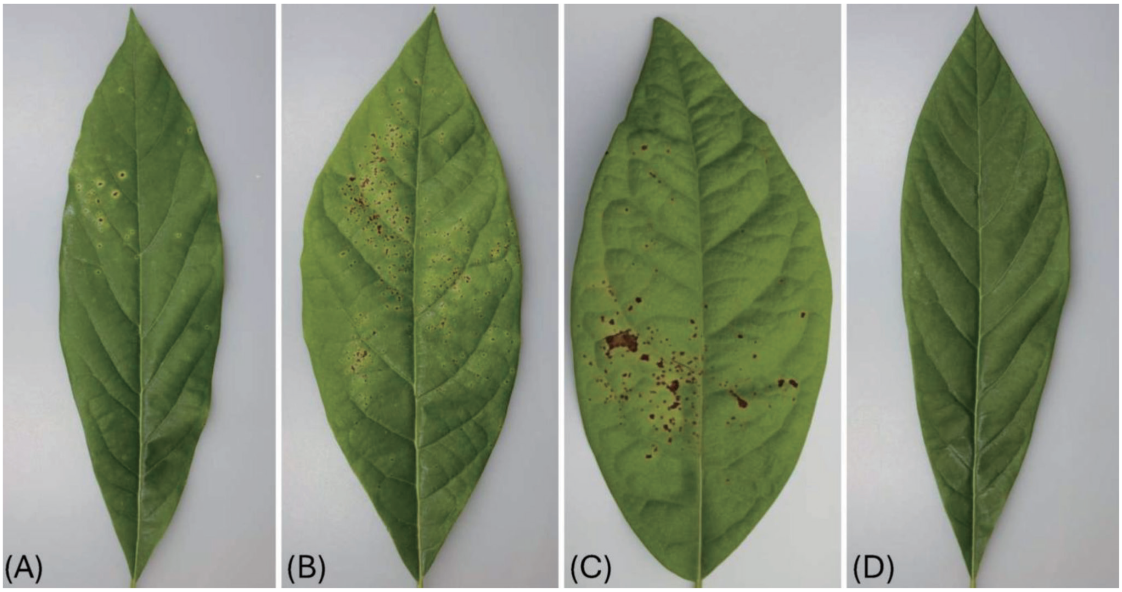

Fig. 4. Leaf symptoms of potted avocado cultivars inoculated with spore suspension of *Pseudoplagiostoma perseae*: (A) 'Hass'; (B) 'Pinkerton'; (C) 'Tainung No. 1 Tasty Red'; and (D) Negative control of a 'Hass' leaf inoculated by sterilized water.

Table 1. Effect of fungicides on mycelial growth of *Pseudoplagiostoma perseae*.

	Inhibition rate (%)[z]					
	1 ppm		10 ppm		100 ppm	
Treatment[y]	V-019	V-027	V-019	V-027	V-019	V-027
23.0% Azoxystrobin SC	50.1 de[x]	50.6 h	35.7 f	46.1 f	38.3 f	44.7 f
62.5% Cyprodinil + Fludioxonil WG	69.0 c	70.1 e	73.1 c	70.9 c	81.4 c	81.9 c
70.0% Thiophanate-methyl WP	67.8 c	62.2 f	100.0 a	100.0 a	100.0 a	100.0 a
23.6% Pyraclostrobin EC	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a
40.0% Iminoctadine-Tris WP	90.9 b	90.7 b	100.0 a	100.0 a	100.0 a	100.0 a
25.9% Tebuconazole EW	89.1 b	79.0 d	100.0 a	100.0 a	100.0 a	100.0 a
80.0% Metiram WG	5.8 f	1.9 i	15.8 g	12.8 g	87.9 b	89.8 b
50.0% Trifloxystrobin WG	51.9 d	58.3 g	52.0 d	58.9 d	57.7 e	62.1 e
32.5% Azoxystrobin + Difenoconazole SC	89.8 b	90.8 b	100.0 a	100.0 a	100.0 a	100.0 a
50.0% Fluopyram + Trifloxystrobin SC	48.2 e	49.8 h	47.4 e	49.5 e	71.7 d	77.7 d
40.0% Thiabendazole WP	90.7 b	89.0 bc	100.0 a	100.0 a	100.0 a	100.0 a
39.5% Fluazinam SC	89.1 b	88.2 c	90.3 b	89.8 b	90.7 b	89.9 b
LSD	2.3	2.4	2.5	2.7	3.6	2.5

[z] Inhibition rate (%) = [(C – T)/C] × 100%, where C is the average colony diameter in the control and T is that in the treatment.
[y] SC: suspension concentrate; WG: water dispersible granules; WP: wettable powder: EC: emulsifiable concentrate: EW: emulsion, oil in water; LSD: least significant difference.
[x] Means within a column followed by the same letter are not significantly different at 5% by Fisher's least significant difference test.

Table 2. Effect of Ffungicides on spore germination of *Pseudoplagiostoma perseae*.

	Spore germination (%)[z]					
	1 ppm		10 ppm		100 ppm	
Treatment[y]	V-019	V-027	V-019	V-027	V-019	V-027
23.0% Azoxystrobin SC	0.0 e[x]	0.0 f	0.0 e	0.0 e	0.0 c	0.0 c
62.5% Cyprodinil + Fludioxonil WG	26.2 c	18.9 d	0.0 e	0.0 e	0.0 c	0.0 c
70.0% Thiophanate-methyl WP	84.2 a	66.7 c	3.5 d	4.2 d	0.0 c	0.0 c
23.6% Pyraclostrobin EC	0.0 e	0.0 f	0.0 e	0.0 e	0.0 c	0.0 c
40.0% Iminoctadine-Tris WP	85.0 a	71.0 b	60.7 b	66.3 b	3.3 b	5.7 b
25.9% Tebuconazole EW	71.5 b	73.2 ab	57.0 c	53.2 c	0.0 c	0.0 c
80.0% Metiram WG	15.5 d	3.7 e	0.0 e	0.0 e	0.0 c	0.0 c
50.0% Trifloxystrobin WG	0.0 e	0.0 f	0.0 e	0.0 e	0.0 c	0.0 c
32.5% Azoxystrobin + Difenoconazole SC	0.0 e	0.0 f	0.0 e	0.0 e	0.0 c	0.0 c
50.0% Fluopyram + Trifloxystrobin SC	0.0 e	0.0 f	0.0 e	0.0 e	0.0 c	0.0 c
40.0% Thiabendazole WP	0.0 e	0.0 f	0.0 e	0.0 e	0.0 c	0.0 c
39.5% Fluazinam SC	0.0 e	0.0 f	0.0 e	0.0 e	0.0 c	0.0 c
CK	83.8 a	75.8 a	83.8 a	75.8 a	83.8 a	75.8 a
LSD	3.0	3.2	2.0	2.3	1.0	1.0

[z] Spore germination (%) = $[(Gc - Gt)/Gc] \times 100\%$, where Gc is the germination rate in the control and Gt is that in the treatment.
[y] SC: suspension concentrate; WG: water dispersible granules; WP: wettable powder; EC: emulsifiable concentrate; EW: emulsion, oil in water; CK: negative control; LSD: least significant difference.
[x] Means within a column followed by the same letter are not significantly different at 5% by Fisher's least significant difference test.

Thailand. In this study, the prevalence period of the disease was investigated in the field. Additionally, the optimal temperature for mycelial growth and spore germination was determined, which reveals the favorable conditions for disease occurrence. Twelve fungicides, registered and recommended in Taiwan, were examined for their ability to inhibit mycelial growth and spore germination. The result provides the growers with guidance on selecting appropriate fungicides for disease management.

In this study, the spore germination rate of *P. perseae* at 20℃ was approximately 63%, which is similar to the rates observed at 25℃ and 30℃. For many pathogenic fungi, such as *Colletotrichum siamense* and *Fusarium oxysporum* (Huang *et al.* 2020; Wu *et al.* 2020), 20℃ is generally not the optimal temperature for spore germination. Instead, temperatures above 25℃ are typically more favorable. Similarly, other pathogenic fungi, including *Neofusicoccum mediterraneum*, *Rhizoctonia solani*, and *Botrytis cinerea* (Thomidis *et al.* 2023; Abouzkhar *et al.* 2024), exhibit optimal mycelial growth between 25℃ and 30℃, and remain active even at 35℃. In contrast, mycelial growth of *P. perseae* ceases at 35℃. These results indicate that elevated temperatures are unfavorable for both spore germination and mycelial growth of *P. perseae*, which may consequently limit its disease development under high-temperature conditions.

For many plant diseases, prevalence is influenced by weather factors. For example, Fusarium head blight on wheat, caused by *Fusarium graminearum*, can be predicted using a logistic regression model that includes three weather-related factors: daily mean relative humidity, daily mean temperature, and the cumulative number of hours with temperatures below 9℃ (Shah *et al.* 2019). Disease outbreaks in host plants may also exhibit seasonal patterns. Grunberg *et al.* (2025) reported that

in the grass species tall fescue (*Lolium arundinaceum*), plant epidemics occurred seasonally, including anthracnose in spring, brown patch in mid-summer, and crown rust from late summer to fall. In this study, the severity of avocado leaf spot disease peaked in the fall and winter. Given that a relatively low temperature (20℃) promotes spore germination of *P. perseae*, and mycelial growth ceases at higher temperatures (35℃), it is reasonable to assume that lower temperatures benefit the development of avocado leaf spot disease. However, a rise in disease severity was observed in July 2021 in Chiayi City (Fig. 1A), suggesting that other factors, such as humidity, rainfall, sunlight intensity, plant cultivars, or cultivation practices, may also contribute to the disease occurrence.

In the fungicide screening assay, six fungicides effectively inhibited mycelia growth, and nine fungicides effectively inhibited spore germination at a concentration of 10 mg a.i. μL^{-1}. The results indicate that *P. perseae* is sensitive to most of the fungicides currently approved and recommended in Taiwan. In the study by Haituk *et al*. (2024), the mycelial growth of *P. perseae* was 100% inhibited by the fungicides mancozeb, carbendazim, prochloraz, and trifloxystrobin, with the inhibition assay for trifloxystrobin conducted at a concentration of 250 mg a.i. μL^{-1}. In this study, the mycelial inhibition rate of trifloxystrobin was only 57% to 62% at a concentration of 100 mg a.i. μL^{-1}. However, trifloxystrobin demonstrated 100% inhibition of spore germination at a concentration of 1 mg a.i. μL^{-1}. Among the twelve fungicides tested, pyraclostrobin and azoxystrobin + difenoconazole effectively inhibit both mycelial growth and spore germination. Carbendazim, thiophanate-methyl, and thiabendazole, which interfere with β-tubulin assembly during mitosis, were found to be effective against *P. perseae*. However, since resistance and cross-resistance to fungicides belonging to benzimidazoles and thiophanates were frequently reported (Putman *et al*. 2010; Lee *et al*. 2011; Fontaine *et al*. 2022), the above three fungicides should be used in rotation with other fungicides to prevent resistance development in the field.

This research provides information on the disease occurrence period in the field and identifies effective fungicides for disease control. However, disease management should be a comprehensive plan that considers both crop cultivation practices and fungicide application. In the future, field assays can be conducted to validate the effectiveness of fungicides in the field. Additionally, studying the impact of crop cultivation practices (such as fertilizer usage, irrigation, and pruning) on disease development would be valuable. The information will help growers establish an integrated management strategy.

CONCLUSION

In this study, field investigations of avocado leaf spot disease were conducted, revealing a seasonal prevalence in fall and winter. The optimal temperature for the mycelial growth of *P. perseae* was found to be 25℃, while the optimal temperature for spore germination ranges from 20℃ to 30℃. Pyraclostrobin completely inhibited the mycelial growth of *P. perseae* at a concentration of 1 mg a.i. μL^{-1}. Complete inhibition of mycelial growth was observed at 10 mg a.i. μL^{-1} with thiophanate-methyl, pyraclostrobin, iminoctadine-tris, azoxystrobin + difenoconazole, and thiabendazole. Spore germination of *P. perseae* is completely inhibited by azoxystrobin, pyraclostrobin, trifloxystrobin, azoxystrobin + difenoconazole, fluopyram + trifloxystrobin, thiabendazole, and fluazinam at a concentration of 1 mg a.i. μL^{-1}. These findings provide important insights into the seasonal dynamics of avocado leaf spot disease and identify effective fungicidal options for its management.

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酪梨葉斑病發生生態及藥劑篩選

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摘要

吳昭蓉、許淑麗、賴素玉。2025。酪梨葉斑病發生生態及藥劑篩選。台灣農業研究 74(4): 449–458。

酪梨葉斑病由真菌 *Pseudoplagiostoma perseae* 所引起，是臺灣酪梨園中常見病害。然而，本病之發生趨勢及有效藥劑之相關知識目前仍十分有限。本研究中，田間調查結果顯示秋、冬兩季為本病好發季節，春天時罹病度則會降低。病原菌菌絲生長之最適溫度為 25℃，孢子發芽最適溫度則為 20–30℃。將病原菌接種於酪梨品種「哈斯」、「平克頓」、「台農一號紅甘」之葉片上，接種 2 wk 後葉片上出現具黃暈之針點斑病徵。藥劑試驗結果，在藥劑濃度 1 mg a.i. L^{-1} 之條件下，百克敏可完全抑制菌絲生長，而亞托敏、百克敏、三氟敏、亞托待克利、三氟派瑞、腐絕及扶吉胺則可完全抑制孢子發芽。本研究之發現，有助於生產者擬定有效的防治策略。

關鍵詞：酪梨、葉斑、病原性、溫度、殺菌劑。

投稿日期：2025 年 5 月 13 日；接受日期：2025 年 7 月 30 日。
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台灣農業研究 (J. Taiwan Agric. Res.) 74(4):459–484 (2025)
https://doi.org/10.6156/JTAR.202512_74(4).0006

Research Article

A Novel Molecular Identification Technique for *Lasiodiplodia* species Associated with Avocado Diseases in Taiwan

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Abstract

Liang, Y. P. and H. F. Ni. 2025. A novel molecular identification technique for *Lasiodiplodia* species associated with avocado diseases in Taiwan. J. Taiwan Agric. Res. 74(4):459–484.

Lasiodiplodia theobromae and *L. pseudotheobromae* are widely distributed plant pathogens in tropical and subtropical regions, causing numerous diseases, but they are not easily identified and distinguished by traditional morphological techniques. In this study, we developed two species-specific primer pairs (Lt-F2/Lt-R1 and Lp-F/Lp-R2) based on sequence differences in translational elongation factor 1-α (*TEF1-α*) among *Lasiodiplodia* species. Polymerase chain reaction (PCR) amplification from genomic DNA verified that Lt-F2/Lt-R1 and Lp-F/Lp-R2 were specific for *L. theobromae* and *L. pseudotheobromae*, respectively; no amplification was observed from nontarget species. Using these primers to amplify DNA from 54 *Lasiodiplodia* isolates from avocado (*Persea americana*) in Taiwan, we successfully identified isolates of *L. theobromae* and *L. pseudotheobromae*. Meanwhile, we also detected the presence of other unknown *Lasiodiplodia* spp. Phylogenetic analyses using the DNA sequences of *TEF1-α*, β-tubulin 2 (*TUB2*), and RNA polymerase subunit II (*RPB2*) from these unknown species showed that *L. thailandica*, *L. hormozganensis*, and other unidentified *Lasiodiplodia* species also occurred on diseased avocado fruits, branches, or pedicels in Taiwan; their pathogenicity to avocado was confirmed on fruits. *L. thailandica* was reported as a pathogen of avocado for the first time, and the specific primers designed in this study provide a valuable tool for identifying the two *Lasiodiplodia* species.

Keywords: *Lasiodiplodia hormozganensis*, *Lasiodiplodia thailandica*, *Lasiodiplodia theobromae*, *Lasiodiplodia pseudotheobromae*, Species-specific primers.

INTRODUCTION

Lasiodiplodia spp. are widely distributed pathogens of woody plants in tropical and subtropical regions, causing numerous diseases (Phillips *et al*. 2013; Castro-Medina *et al*. 2014; Netto *et al*. 2014; Pipattanapuckdee *et al*. 2019). Among several *Lasiodiplodia* spp. associated with avocado (*Persea americana*), *L. theobromae* was reported to cause avocado branch canker, dieback, and stem end rot in Chile and Thailand (Trakunyingcharoen *et al*. 2015a; Valencia *et al*. 2019), dieback in Peru and Spain (Arjona-Girona *et al*. 2019; Rodríguez-Gálvez *et al*. 2021), branch canker in California (Avenot *et al*. 2023), postharvest fruit rot in Italy (Garibaldi *et al*. 2012), and branch blight in China (Qiu *et al*. 2020). In addition, *L. pseudotheobromae* has been demonstrated to cause avocado branch canker and stem-end

Received: June 6, 2025; Accepted: August 25, 2025.
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rot in Thailand and dieback in Peru (Trakunyingcharoen *et al*. 2015a; Rodríguez-Gálvez *et al*. 2021). Moreover, *L. laeliocattleyae* and *L. citricola* were reported as pathogens causing avocado dieback in Peru and Italy, respectively (Rodríguez-Gálvez *et al*. 2021; Fiorenza *et al*. 2023), and *L. euphorbiaceicola* and *L. mahajangana* in Hainan, China (Chen *et al*. 2024), and *L. hormozganensis* in Sri Lanka (Nilmini *et al*. 2020) were reported as pathogens causing avocado stem-end rot. As mentioned above, *L. theobromae* and *L. pseudotheobromae* were the most frequently reported, and also were proved to be important pathogens causing branch canker and stem-end rot of avocado in Taiwan (Liang *et al*. 2021).

Due to their similar morphologies, *L. pseudotheobromae* was often misidentified as *L. theobromae* prior to the work of Alves *et al*. (2008), who distinguished them using a combination of morphological and phylogenetic analyses. Although some morphological and culture characteristics were suggested for distinguishing *L. pseudotheobromae* from *L. theobromae* (Alves *et al*. 2008), as more isolates from different geographic areas have been examined, has been called into question whether these characteristics are particular to *L. pseudotheobromae* (Liang *et al*. 2021). Because morphological and culture characteristics are not sufficient to distinguish between these species and also other closely related species of *Lasiodiplodia*, it is essential to use sequence-based identification based on multiple loci (Cruywagen *et al*. 2017; Rathnayaka *et al*. 2023). Sequences of the internal transcribed spacer (ITS) of genomic ribosomal DNA (rDNA) and the nuclear small subunit (SSU) rRNA, nuclear large subunit (LSU) rRNA, translational elongation factor 1-α (*TEF1-α*), β-tubulin 2 (*TUB2*), RNA polymerase subunit II (*RPB2*), and calmodulin (*cmdA*) genes have been used for phylogenetic analyses and identification of *Lasiodiplodia* spp. (Alves *et al*. 2008; Endes *et al*. 2016; Cruywagen *et al*. 2017; Li *et al*. 2019; Wang *et al*. 2021; Ko *et al*. 2023; Rathnayaka *et al*. 2023). However, identification using multi-locus phylogenetic analyses can be time-consuming and expensive.

Additionally, different *Lasiodiplodia* species can co-occur on the same plant and cause similar symptoms, further complicating identification (Ko *et al*. 2023). For example, both *L. theobromae* and *L. pseudotheobromae* have been demonstrated to cause branch canker and stem-end rot of avocado (Trakunyingcharoen *et al*. 2015a; Liang *et al*. 2021), dieback and stem-end rot of mango (*Mangifera indica*) (Ismail *et al*. 2012; Marques *et al*. 2013), and stem-end rot of papaya (*Carica papaya*) (Netto *et al*. 2014). A study by Rathnayaka *et al*. (2023) suggested that *L. theobromae* is the predominant species in tropical and subtropical regions and that *L. pseudotheobromae* is the second most prevalent species. Given their prevalence and economic importance, developing a cost-effective method to identify these species from non-purified cultures could facilitate epidemiological studies.

One cost-effective method that has been widely used for the accurate identification of fungal pathogens is polymerase chain reaction (PCR) with species-specific primers (Hariharan & Prasannath 2021). Although a species-specific primer pair for *L. theobromae* was developed in a previous study (Ni *et al*. 2012), it failed to distinguish *L. pseudotheobromae* from *L. theobromae*, as it amplified a PCR product of a similar size from both species. Therefore, we aimed to develop species-specific primers for cost-effective identification of these two species using PCR. Subsequently, these primers were used to identify *Lasiodiplodia* isolates from avocado in Taiwan. For some isolates, no product could be amplified by either pair of primers. Therefore, we next identified these isolates by performing multi-locus phylogenetic analyses and assessing their pathogenicity to avocado.

MATERIALS AND METHODS

DNA extraction

Mycelia were collected from colonies cultivated on potato dextrose agar (PDA, Difco Inc., Detroit, MI, USA). All DNA was extract-

ed using QuickExtract™ Plant DNA Extraction Solution (Epicenter, Madison, WI, USA), except for the DNA used in the primer sensitivity test, which was extracted using MasterPure yeast DNA purification kit (Lucigen, Middleton, WI, USA) and quantified using a microplate spectrophotometer (Epoch, Bio Tek Instruments, Inc., Winooski, VT, USA). Purified genomic DNA was stored at -20℃ until use.

Design of *L. theobromae*- and *L. pseudotheobromae*-specific primers

Eight isolates of *L. theobromae* and five isolates of *L. pseudotheobromae* were selected for designing species-specific primers (Appendix 1). The *TEF1-α* sequences of these isolates retrieved from NCBI (http://www.ncbi.nlm.nih.gov/) (Appendix 1) were analyzed by multiple sequence alignment using Vector NTI Advance 11.5 (Invitrogen, Carlsbad, CA, USA). Two primer pairs were first designed based on the sequence variability between *L. theobromae* and *L. pseudotheobromae*: Lt-F2 (5′-AGTTCGAGAAGGTCCGTGCAC-3′), Lt-R1 (5′-TTCCTGTAGTGGGGCGC-3′), Lp-F (5′-AACCCCGCTTGGCTCTGT-3′), and Lp-R2 (5′-GTCCATCTTGTTGATGGCGAC-3′). The predicted sizes of the products amplified by primer pairs Lt-F2/Lt-R1 and Lp-F/Lp-R2 were 272 bp and 567 bp for *L. theobromae* and *L. pseudotheobromae*, respectively. To further evaluate the specificity of the primers, the sequences of *TEF1-α* at the primer annealing sites in *L. theobromae* and *L. pseudotheobromae* were compared with those of 31 other *Lasiodiplodia* spp., each represented by a single isolate (Appendix 1).

Assessment of primer specificity and sensitivity

Because only *L. theobromae* and *L. pseudotheobromae* have been reported on avocado in Taiwan previously, the isolates used for the specificity test included 6 *L. theobromae* isolates, 4 *L. pseudotheobromae* isolates, and other Botryosphaeriaceae spp. isolated from avocado, including *Neofusicoccum parvum* B1118, *N. parvum* B1174, *Neofusicoccum mangiferae* B1183, *N. mangiferae* B1640, *Botryosphaeria dothidea* B1134, and *B. dothidea* B1147 (Ni *et al.* 2011). PCR was performed in a 25-μL reaction mixture containing 5 μL of Fast-Run^{TM} Taq Master Mix 5× (Protech Technology Enterprise Co., Ltd., Taipei, Taiwan), 18 μL of ddH_2O, 0.5 μL of each primer (10 pmole μL^{-1}), and 1 μL of DNA template. The PCR conditions were 4 min of initial denaturation at 94℃, followed by 31–33 cycles of 30 s of denaturation at 94℃, 30 s of annealing at 51–68℃, and 30 s of extension at 72℃, and a final extension of 10 min at 72℃. The optimum annealing temperature was empirically investigated by testing temperatures ranging from 51–68℃. The sensitivity of each primer pair was analyzed by using a serial dilution of purified fungal DNA as the template for PCR. Given the difference in expected amplicon sizes, the two primer pairs were also combined in a single PCR reaction to assess their suitability for multiplex PCR applications. The amplified products were stained with EZ-Vision® Three DNA Dye, and analyzed by 1.5% agarose gel electrophoresis in Tris-borate-EDTA buffer.

Identification of *Lasiodiplodia* isolates from avocado using species-specific primers

Lasiodiplodia isolates were isolated from avocado fruits with stem-end rot and branches with cankers between 2018–2020. The symptomatic tissues were disinfected by immersion in 0.5% NaClO for 30 s, rinsed with sterile water, and then placed in Petri dishes containing acidified potato dextrose agar [APDA, 300 mL PDA with 750 μL 50% (v/v) lactic acid]. The plates were incubated at 25℃ for 1 to 4 d. Colonies were transferred to 2% water agar, and single hyphal tips were transferred to PDA to obtain a pure culture. The DNA of each isolate was extracted and subjected to PCR using the species-specific primer pairs developed in this study as aforementioned.

Identification of *Lasiodiplodia* isolates from avocado by phylogenetic analyses

To identify the *Lasiodiplodia* isolates used in this study, phylogenetic analyses were conducted using partial sequences of the *TEF1-α*, *TUB2*, and *RPB2* genes because they were suggested as reliable molecular markers for the identification of *Lasiodiplodia* (Rathnayaka *et al*. 2023). The *TEF1-α*, *TUB2*, and *RPB2* nuclear gene regions were amplified using primers EF688F and EF1251R (Alves *et al*. 2008) or EF1F and EF2R (for B2885 and B2900) (Jacobs *et al*. 2004), Bt2a and Bt2b (Glass & Donaldson 1995), and rpb2-LasF and rpb2-LasR (Cruywagen *et al*. 2017), respectively. The PCR reaction mix was prepared as aforementioned. The PCR product was purified and sequenced by Tri-I Biotech, Inc. (Taipei, Taiwan).

Bayesian inference was used to construct phylogenetic trees. Sequences of 96 *Lasiodiplodia* isolates from the GenBank database and this study were included in the trees (Appendix 1). For each gene, a multiple sequence alignment was conducted using ClustalX v.2.1 (Larkin *et al*. 2007), and these alignments were concatenated using SequenceMatrix v.2.1.10 (Vaidya *et al*. 2011). jModelTest (Darriba *et al*. 2012) was used to select the best-fit DNA substitution model under the Bayesian information criterion. The DNA substitution models used for *TEF1-α*, *TUB2*, and *RPB2* were HKY + I + G, HKY + I, and K80 + I, respectively. For confirming the identification of *L. theobromae* and *L. pseudotheobromae* using species-specific primers, only the partial sequences of *TEF1-α* and *TUB2* were used to construct a phylogenetic tree. The DNA substitution models used for *TEF1-α* and *TUB2* were HKY + I + G and HKY + G, respectively. Phylogenetic tree construction was conducted with MrBayes v.3.2.6 (Ronquist *et al*. 2012). The analyses were run for 1.5×10^7 generations, and samples were taken from the posterior every 15,000 generations. The first 25% of generations were discarded as a burn-in period. Both trees were rooted with *Diplodia seriata* CBS112555.

Morphological characterization

Four isolates of *L. hormozganensis* (B2828, B2831, B2847, and B2858), 5 isolates of *L. thailandica* (ASS65, B2844, B2848, B2870, and B2913), and 8 isolates of unknown *Lasiodiplodia* spp. (AZP74, B2850, B2881, B2903, B2915, B2917, B2928, and B2930) were selected for morphological characterization. Colony morphology was characterized by growing isolates on 9-mm Petri dishes containing PDA for 3 d at 25℃ and 14 d at 35℃ in darkness. Pycnidia and conidia were induced by placing mycelial plugs on 2% (w/v) water agar (Merck, Darmstadt, Germany) supplemented with autoclaved horsetail stem (*Casuarina equisetifolia* L.) (Ni *et al*. 2011; Kee *et al*. 2017) and incubating for over 21 d at 25℃ under black light (TL-D 18W BLB, Philips, Amsterdam, The Netherlands). Morphological characteristics of conidia (e.g., color, shape, and septation) were observed with a Nikon 80i microscope (Tokyo, Japan), and pictures were captured with a Progres Gryphax camera (Jenoptik, Jena, Germany). The length and width of 50 conidia were measured.

Pathogenicity test on fruits

L. hormozganensis B2828, *L. pseudotheobromae* B2854, *L. thailandica* B2848, *L. theobromae* B2891, and *Lasiodiplodia* sp. B2903 were used in the pathogenicity test. 'Choquette' fruits were harvested at maturity and surface disinfected with 75% ethanol. The fruits were wounded with a flame-sterilized needle (5 mm in depth), and 2-day-old mycelial plugs (5 mm in diameter) of the isolates were placed on the wounds with the mycelial side facing the fruits. Control fruits were treated with sterile PDA discs (5 mm in diameter). There were 5 replicates for each isolate. The fruits were kept at 25℃ in 100% relative humidity for the first 2 d after inoculation, and then the relative humidity was reduced to 65% for the remainder of the trial. The diameters of the necrotic lesions on fruits were measured at 7 d after inoculation. Statistical analysis was conducted using SAS Enterprise Guide version 7.15 (SAS

Institute Inc., Cary, NC, USA). Before conducting a one-way analysis of variance (ANOVA), a Levene's test was performed to assess the assumption of homogeneity of variance. The results were non-significant (P = 0.31), indicating that the assumption of equal variances was met. Subsequently, the data were subjected to one-way ANOVA followed by Fisher's least significant difference test. Pathogens were re-isolated from lesions, and *L. pseudotheobromae* B2854 and *L. theobromae* B2891 were re-identified by PCR with species-specific primers, while *L. hormozganensis* B2828, *L. thailandica* B2848, and *Lasiodiplodia* sp. B2903 were re-identified by analysis of *TEF1-α* sequences. The experiment was replicated twice.

RESULTS

Design and assessment of *L. theobromae*- and *L. pseudotheobromae*-specific primers

PCR was conducted with 31 cycles, and the optimal annealing temperatures for the Lt-F2/Lt-R1 and Lp-F/Lp-R2 primer pairs were 59℃ and 60℃, respectively. The primer pairs Lt-F2/Lt-R1 and Lp-F/Lp-R2 amplified a single PCR band of the expected size from *L. theobromae* and *L. pseudotheobromae* isolates, respectively, but not from other Botryosphaeriaceae spp. (Fig. 1). The expected amplicon sizes were 272 bp and 567 bp, respectively. Primer sensitivity was analyzed by using a serial dilution of purified fungal DNA as the template for PCR. Analysis of the amplified products by agarose gel electrophoresis indicated that the detection limit of PCR was approximately 1 ng for each of the primer pairs (Fig. 2).

To further evaluate the specificity of the primers, the sequences of *TEF1-α* at the primer annealing sites of *L. theobromae* and *L. pseudotheobromae* were compared with those of other *Lasiodiplodia* spp. (Fig. 3). The alignment of *TEF1-α* sequences showed that the Lt-F2 sequence was unique to *L. theobromae*. Because there was insufficient sequence information for *L. viticola* CBS 128313, the *TEF1-α* sequences of two other *L. viticola* isolates with more sequence information were analyzed. The results showed that the sequence of *L. viticola* CMW40944 (GenBank accession number KP872376) differed from that of *L. theobromae* at this site, while that of *L. viticola* CMM1472 (GenBank accession number JX464040) was the same

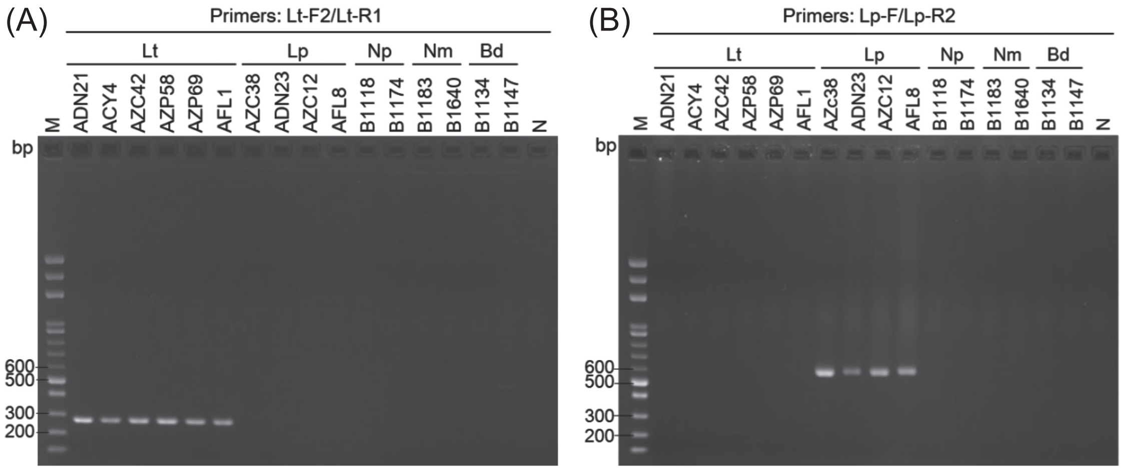

Fig. 1. Polymerase chain reaction (PCR) amplified products with species-specific primer pairs (A) Lt-F2/Lt-R1 and (B) Lp-F/Lp-R2. M: 100-bp DNA ladder (Protech Technology Enterprise, Taipei, Taiwan). N: negative control. Abbreviations: Lt: *Lasiodiplodia theobromae*; Lp: *Lasiodiplodia pseudotheobromae*; Np: *Neofusicoccum parvum*; Nm: *Neofusicoccum mangiferae*; Bd: *Botryosphaeria dothidea*.

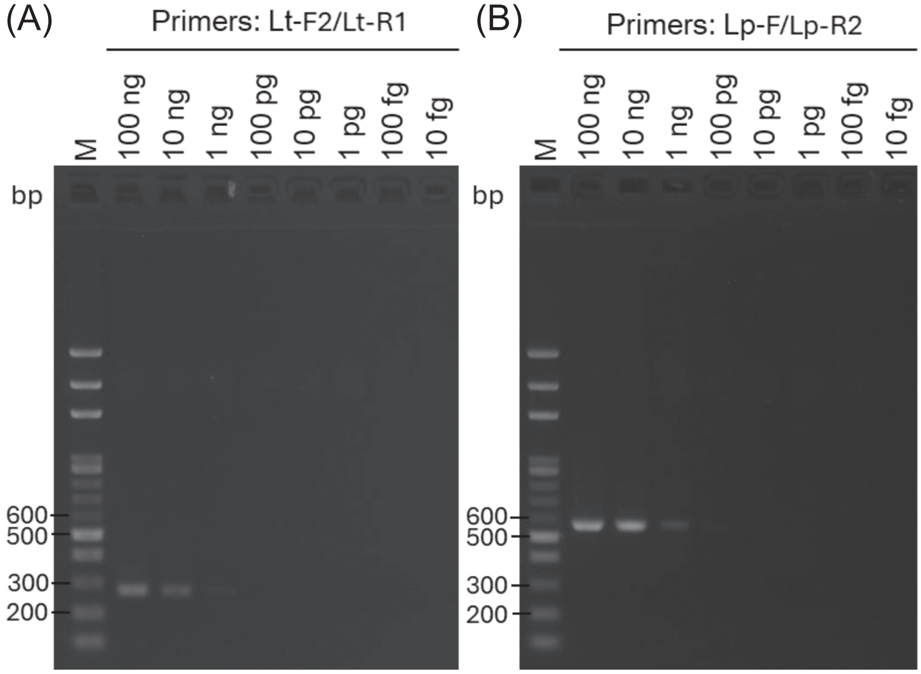

Fig. 2. Sensitivities of (A) Lt-F2/Lt-R1 and (B) Lp-F/Lp-R2 determined using 10-fold serial dilutions of *L. theobromae* ADN21 and *L. pseudotheobromae* AZC38 genomic DNA, respectively, ranging from 100 ng to 10 fg, as templates. M, 100-bp DNA ladder (Protech Technology Enterprise, Taipei, Taiwan).

(data not shown). In the region corresponding to Lt-R1, *L. mahajangana* CMW27801, *L. viticola* CBS128313, *L. bruguierae* CMW 41470, *L. chonburiensis* MFLUCC 16-0376, and *L. gilanensis* IRAN1523C shared the identicalsequences with *L. theobromae*. The Lp-F sequence was unique to *L. pseudotheobromae*. Most of the *TEF1-α* sequences of *Lasiodiplodia* spp. in NCBI lack information about the sequence from which Lp-R2 was designed. Among the species with such details, *L. theobromae* AZP58, *L. mahajangana* CMW27801, and *L. euphorbiacieco-la* CMM3609 shared the same sequence as *L. pseudotheobromae* (Fig. 3).

Although the sizes of the amplified products differed, the two primer pairs amplified two PCR products (200–300 bp and 500–600 bp) from *L. theobromae* DNA when combined (Appendix 3). Therefore, these primer pairs are not suitable for multiplex PCR. It is probably because there was at most one-nucleotide difference between *L. theobromae* and *L. pseudotheobromae* at the annealing site of Lp-R2, so Lt-F2/Lp-R2 could also amplify an amplicon from *L. theobromae* DNA (Appendix 3).

Identification of *Lasiodiplodia* isolates from avocado using species-specific primers

A total of 54 *Lasiodiplodia* isolates were collected from avocado fruits, pedicels, and branches in 2018–2020 (Appendix 2). Among them, 16 isolates (B2852, B2854, B2855, B2857, B2864, B2866, B2871, B2876, B2880, B2885, B2888, B2894, B2895, B2942, B2944, and B2946) were amplified by Lp-F/Lp-R2 with a 500–600 bp product, indicating that they were *L. pseudotheobromae*, while 19 isolates (B2825, B2827, B2843, B2849, B2862, B2863, B2865, B2873, B2875, B2877, B2886, B2887, B2891, B2900, B2926, B2937, B2940, B2943, B2953) were amplified by Lt-F2/Lt-R1 with a 200–300 bp product, indicating that they were *L. theobromae* (Fig. 4).

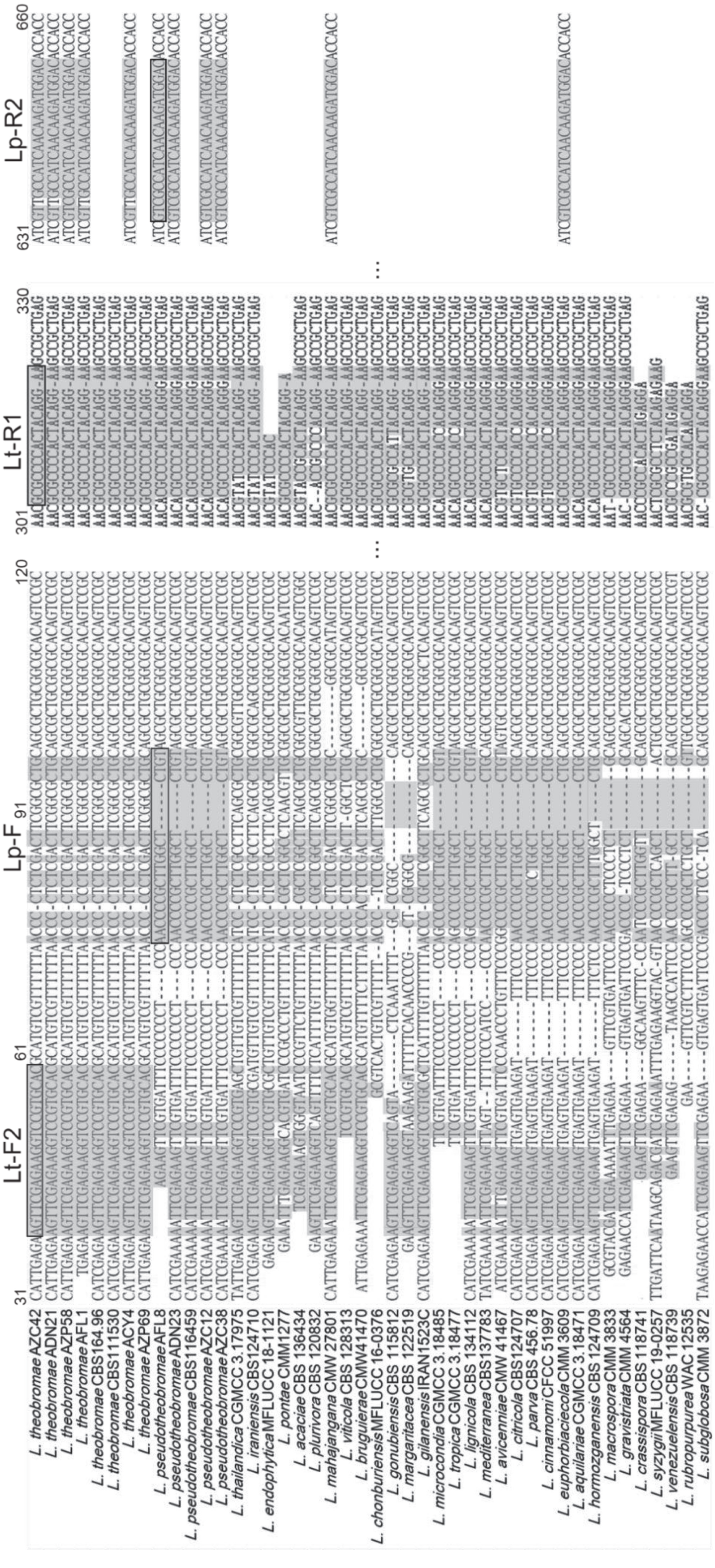

Fig. 3. Nucleotide sequence alignment of the translation elongation factor 1-α genes from different *Lasiodiplodia* species. Positions of the primers for species-specific detection of *L. theobromae* and *L. pseudotheobromae* are boxed with rectangles. Sequences that are identical to the primers are shaded in gray.

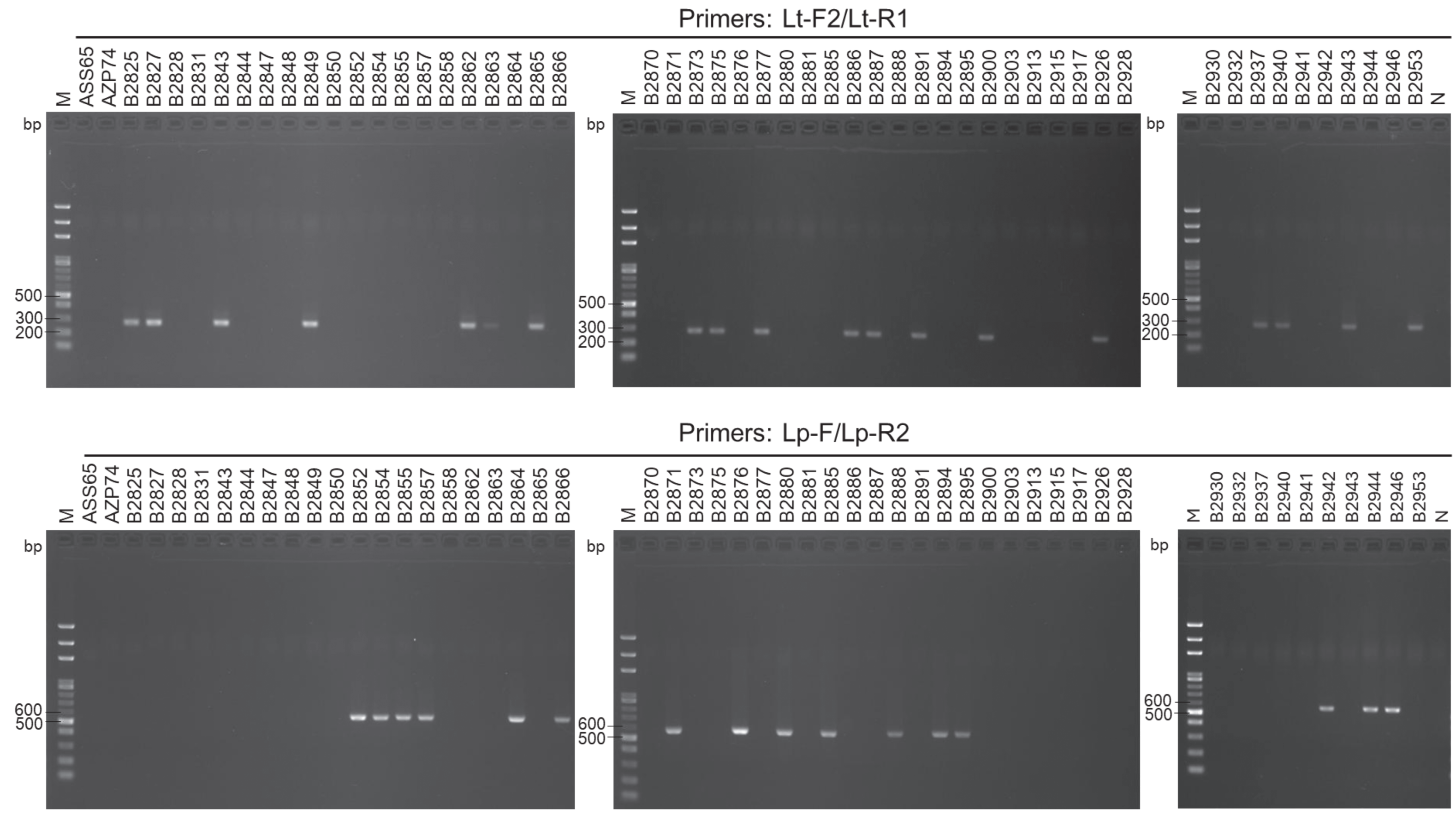

Fig. 4. Identification of *Lasiodiplodia* isolates from avocado using species-specific primers pairs Lt-F2/Lt-R1 (top) and Lp-F/Lp-R2 (bottom). M: 100-bp DNA ladder (Protech Technology Enterprise, Taipei, Taiwan). N: negative control.

To confirm the PCR-based identification of these isolates, the partial *TEF1-α* and *TUB2* sequences of these isolates and 73 isolates from the GenBank database were concatenated to form a supermatrix of 1,267 bp and used for phylogenetic analysis (Appendix 1). In this tree, B2852, B2854, B2888, B2866, B2855, B2857, B2864, B2871, B2876, B2880, B2885, B2894, B2895, B2942, B2944, and B2946 were clustered with *L. pseudotheobromae*, while B2825, B2827, B2843, B2849, B2862, B2863, B2865, B2873, B2875, B2877, B2886, B2887, B2891, B2900, B2926, B2953, B2937, B2940, and B2943 were clustered with *L. theobromae*, with Bayesian posterior probability (BPP) values of 69% and 100%, respectively, confirming the results obtained by PCR with Lt-F2/Lt-R1 and Lp-F/Lp-R2 (Appendix 4).

Discovery of novel *Lasiodiplodia* pathogens on avocado

No PCR product was amplified from 19 isolates (ASS65, AZP74, B2828, B2831, B2844, B2847, B2848, B2850, B2858, B2870, B2881, B2903, B2913, B2915, B2917, B2928, B2930, B2932, and B2941) by the 2 primer pairs (Fig. 4). Therefore, these isolates were subjected to phylogenetic analysis.

To identify these isolates, DNA sequences of the three gene regions (*TEF1-α*, *TUB2*, and *RPB2*) from these isolates, *L. pseudotheobromae* B2854 and B2885, *L. theobromae* B2891 and B2900, and 73 isolates from the GenBank database were concatenated to form a supermatrix of 1,918 bp and used for phylogenetic analysis (Appendix 1). The results showed that 4 isolates (B2828, B2831, B2847, and B2858) were clustered with *L. hormozganensis*, while 7 isolates (ASS65, B2844, B2848, B2870, B2913, B2932, and B2941) were in the *L. thailandica* clade; in both cases the BPP value was 100%. Eight isolates (AZP74, B2850, B2858, B2903, B2915, B2917, B2928, and B2930) clustered with *L. chiangraiensis*, *L. endophytica*, *L. ireniensis*, and *L. thailandica*, but their taxonomical positions could not be determined (Fig. 5).

To confirm the phylogenetic analysis-based identification, the morphological characteristics of the 19 isolates that did not yield a PCR product with the species-specific primers were examined. All isolates grew rapidly on PDA, covering the entire surface of the Petri dishes within 3 d. Colonies of all isolates were gray-white on the upper surface with fluffy aerial mycelia, and for some of the isolates, the middle of the colonies turned grayish-gray (Appendix 5). When cultured in PDA at 35℃, none of the 4 *L. hormozganensis* isolates produced red pigments, while all 7 of the *L. thailandica* isolates produced a red pigment, ranging in color from light pink to deep crimson (Appendix 6). Among the 8 isolates of unidentified *Lasiodiplodia* spp., AZP74, B2903, B2928, and B2930 showed the production of red pigments, while B2850, B2881, B2915, and B2917 did not. All isolates formed pycnidia and conidia on horsetail stems on water agar within 2–4 wk. Conidia of all isolates were subovoid to ellipsoid, smooth, initially hyaline and aseptate, becoming dark brown, 1-septate, and striated when mature. Conidia of *L. thailandica* isolates were 17–29 × 10–16 μm, L/W = 1.7–1.9 (n = 50) (Table 1, Appendix 7), while those of *L. hormozganensis* isolates were 15–24 × 10–14 μm, L/W = 1.4–1.9 (n = 50) (Table 1, Appendix 7). The conidia of unidentified *Lasiodiplodia* spp. isolates were 19–29 × 10–16 μm, L/W = 1.7–2.0 (n = 50) (Table 1, Appendix 7). No sexual (teleomorph) structures were observed during this study.

Pathogenicity test on fruits

To confirm that the *Lasiodiplodia* spp. isolated in this study were causal agents of avocado stem-end rot, pathogenicity tests were conducted on avocado fruits. The tests showed that *L. hormozganensis* B2828, *L. pseudotheobromae* B2854, *L. thailandica* B2848, *L. theobromae* B2891, and *Lasiodiplodia* sp. B2903 caused black lesions on avocado fruits. The external lesions were visible at 2 d after inoculation and continued to grow larger. Underneath the lesions, the pulp turned soft, water-soaked,

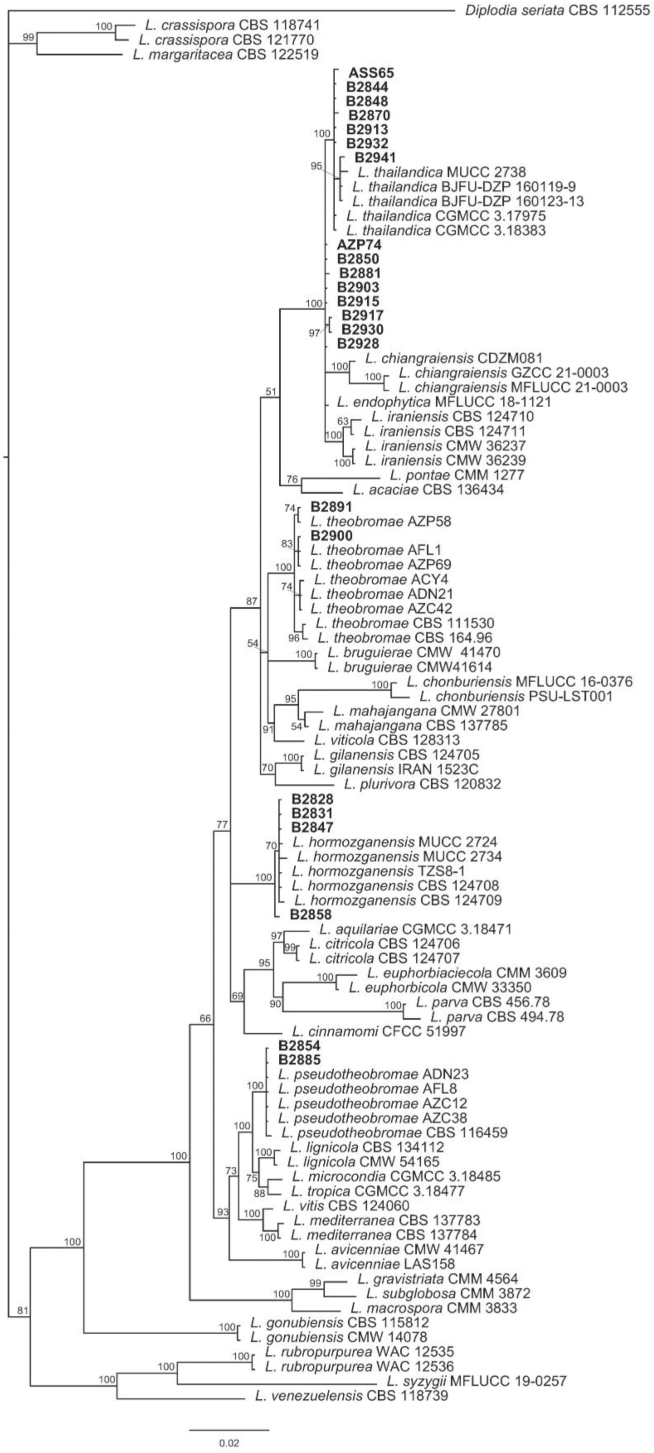

Fig. 5. Bayesian phylogenetic tree of *Lasiodiplodia* isolates from avocado in Taiwan and from the GenBank database. The phylogenetic tree was built using concatenated sequences of partial *TEF1-α*, *TUB2*, and *RPB2* gene regions. The Bayesian posterior probabilities are indicated next to the nodes. The tree is rooted with *Diplodia seriata* CBS 112555. Isolates from this study are emphasized in bold.

Table 1. Morphological characteristics of the *Lasiodiplodia* isolates examined in this study.

Species	Isolate	Conidial size (μm) Length	Width	L/W[z]	Colony pigmentation at 35℃
L. hormozganensis	B2828	15.2–18.3–21.7	10.4–12.4–13.9	1.48	None
L. hormozganensis	B2831	19.1–21.4–23.5	9.3–12.1–13.7	1.78	None
L. hormozganensis	B2847	19.0–21.1–23.9	11.1–12.2–13.4	1.74	None
L. hormozganensis	B2858	17.6–20.8–23.6	10.0–11.3–12.7	1.83	None
L. thailandica	ASS65	20.9–23.8–27.6	10.9–12.9–15.4	1.86	Red
L. thailandica	B2844	19.7–23.5–27.2	10.7–13.7–15.3	1.73	Slightly pink
L. thailandica	B2848	18.9–23.2–26.4	10.8–13.2–14.5	1.77	Slightly pink
L. thailandica	B2870	21.0–24.0–28.8	11.4–13.9–15.6	1.73	Pink
L. thailandica	B2913	19.9–23.2–27.3	10.7–12.3–15.0	1.89	Slightly pink
L. thailandica	B2941	18.4–23.3–26.8	11.2–13.3–15.2	1.75	Pink
L. thailandica	B2932	17.2–23.2–27.4	10.3–12.6–14.3	1.85	Pink
Lasiodiplodia sp.	AZP74	22.1–23.8–25.7	10.3–12.4–15.6	1.93	Pink
Lasiodiplodia sp.	B2850	19.6–24.9–28.0	12.1–13.3–15.1	1.87	None
Lasiodiplodia sp.	B2881	21.5–24.9–27.7	11.0–12.6–14.4	1.98	None
Lasiodiplodia sp.	B2903	19.9–22.6–26.0	10.5–12.9–14.5	1.76	Pink
Lasiodiplodia sp.	B2915	18.8–23.1–26.8	11.5–12.6–14.6	1.84	None
Lasiodiplodia sp.	B2917	18.8–22.6–26.9	10.5–12.1–13.3	1.87	None
Lasiodiplodia sp.	B2928	19.1–24.6–28.2	11.9–13.8–16.1	1.78	Pink
Lasiodiplodia sp.	B2930	20.5–23.8–27.0	10.7–12.3–14.1	1.93	Slightly pink

[z] L/W: average length/average width.

and black (Appendix 8). The average diameters of the lesions caused by *L. hormozganensis* B2828, *L. thailandica* B2848, and *L. theobromae* B2891 were all more than 100 mm, which is significantly larger than the lesions caused by *L. pseudotheobromae* B2885 and *Lasiodiplodia* sp. B2903 (*P*-value < 0.0001) (Table 2). All control fruits remained symptomless at the treatment sites, and no fungi were re-isolated. All of the isolates were successfully re-isolated from necrotic pulps and re-identified based on species-specific primers or *TEF1-α* sequences, and thus, Koch's postulates were fulfilled.

Table 2. Pathogenicity tests on 'Choquette' fruits 7 d post inoculation.

Isolates	Lesion diameter (mm, mean ± *SD*)[z]
L. hormozganensis B2828	133.6 ± 13.6 a
L. pseudotheobromae B2885	95.7 ± 15.7 b
L. thailandica B2848	123.7 ± 11.9 a
Lasiodiplodia sp. B2903	95.1 ± 14.6 b
L. theobromae B2891	137.6 ± 15.9 a
Control	0.0 ± 0.0 c
P-value	< 0.0001

[z] Mean and standard deviation (*SD*) are derived from 5 replicates. Means in the same column followed by the same letter are not significantly different according to Fisher's least significant difference test ($P = 0.05$). There were five replicates for each isolate.

DISCUSSION

Accurate and cost-effective identification of plant fungal pathogens is crucial for disease management, as their sensitivity to fungicides differ slightly (Chen *et al.* 2024). Because *L. theobromae* and *L. pseudotheobromae* are two of the most prevalent *Lasiodiplodia* species in tropical and subtropical regions (Rathnayaka *et al.* 2023) and are frequently isolated from

avocado and difficult to distinguish by morphological and culture characteristics (Alves *et al*. 2008; Liang *et al*. 2021), it is necessary to develop a molecular method for prompt identification. Toward this end, we developed 2 pairs of species-specific primers based on the differences in *TEF1-α* sequences between *L. theobromae* and *L. pseudotheobromae*, and these primers proved to be helpful in distinguishing and identifying these 2 species.

In addition, the primers we developed can detect *L. theobromae* and *L. pseudotheobromae* from a DNA mixture of these 2 species (unpublished data). Therefore, even when these species co-occur on the same plant tissue, diagnosis can be conducted by performing PCR with species-specific primers using DNA directly extracted from mycelia growing from the plant tissues without purifying the fungal isolates first.

Because no DNA from other *Lasiodiplodia* spp. was available when testing the specificity of the primer pairs, the *TEF1-α* sequences of 31 other *Lasiodiplodia* spp., each represented by 1 isolate, were compared at the primer annealing sites. The results showed that the *TEF1-α* sequence of *L. viticola* CMM1472 at both the Lt-F2 and Lt-R1 sites was the same as that of *L. theobromae*. Besides, *L. microconidia*, *L. lignicola*, and *L. tropica* only have one nucleotide difference at the Lp-F site from *L. pseudotheobromae*, and the phylogenetic analyses showed that they were also closely related to it. Although the possibility that the primer pairs might also amplify a product from these *Lasiodiplodia* species cannot be ruled out, the identities of the 19 isolates identified as *L. theobromae* by Lt-F2/Lt-R1 and 16 isolates identified as *L. pseudotheobromae* by Lp-F/Lp-R2 in this study were confirmed based on phylogenetic analysis of partial *TEF1-α* and *TUB2* sequences. Because of the similarity between available DNA sequences of all *Lasiodiplodia* species, it is difficult to design a single primer pair to distinguish *L. theobromae* from all other species. Further studies could select other gene sequences for designing a second primer pair to verify identification.

Using Lt-F2/Lt-R1 and Lp-F/Lp-R2 to screen 54 *Lasiodiplodia* isolates from avocado revealed that most *Lasiodiplodia* isolates from avocado in Taiwan are either *L. theobromae* or *L. pseudotheobromae*. Moreover, phylogenetic analyses revealed that the remaining isolates were *L. thailandica*, *L. hormozganensis*, and some unidentified *Lasiodiplodia* spp. These results suggest that primer pairs Lt-F2/Lt-R1 and Lp-F/Lp-R2 can be used not only to identify *L. theobromae* and *L. pseudotheobromae* but also to distinguish them from other *Lasiodiplodia* spp. associated with avocado.

Lasiodiplodia has a global distribution and occurs on a wide range of hosts (Ko *et al*. 2023; Rathnayaka *et al*. 2023). In recent years, multi-locus phylogenetic analyses combined with morphological characteristics have been used to re-examine previously identified *Lasiodiplodia* isolates, revealing cryptic species (Hattori *et al*. 2023; Ko *et al*. 2023). We found that the specific primers Lt-F2/Lt-R1 and Lp-F/Lp-R2 could identify *L. theobromae* and *L. pseudotheobromae* not only from avocado but also from other hosts (unpublished data), which means researchers could use these primers to screen through their collections of *Lasiodiplodia* spp. and identify isolates of *L. theobromae* and *L. pseudotheobromae* first, and then the remaining isolates could be subjected to further analysis. Therefore, these primers could be useful and cost-effective tools for investigating the diversity of *Lasiodiplodia* spp.

The pathogenicity tests in this study showed that *L. thailandica* B2848, *L. hormozganensis* B2828, and *Lasiodiplodia* sp. B2903 were all pathogenic to avocado fruits. *L. thailandica* was first reported on *M. indica* and *Phyllanthus acidus* in Thailand (Trakunyingcharoen *et al*. 2015b). Since then, this species has been reported to be associated with *Podocarpus macrophyllus* and *Albizia chinensis* in China, *M. indica* and *Kalanchoe pinnata* in Japan, and a *Cerasus* sp. in

Taiwan (Dou *et al*. 2017; de Silva *et al*. 2019; Hattori *et al*. 2023; Rathnayaka *et al*. 2023), but it has not previously been reported as a pathogen of avocado. The reported conidial dimensions of *L. thailandica* were (20–)22–25(–26) × (12–)13–15(–16) µm, and those of the *L. thailandica* isolates identified in this study were all within this range, confirming the identification from phylogenetic analysis. To our knowledge, this is the first study describing the pigmentation of *L. thailandica* at 35℃, and demonstrating that *L. thailandica* is also a pathogen causing avocado stem-end rot disease.

L. hormozganensis has been reported to be associated with avocado in Sri Lanka (Nilmini *et al*. 2020), *M. indica* and an *Olea* sp. in Iran (Abdollahzadeh *et al*. 2010), *C. papaya*, *M. indica*, and *Ricinus communis* in Brazil (Abdollahzadeh *et al*. 2010; Marques *et al*. 2013; Netto *et al*. 2014), several herbaceous and woody plants in Japan (Hattori *et al*. 2023), and *Syzygium samarangense*, *M. indica*, *Psidium guajava*, *Annona squamosal*, and *Musa* spp. in Taiwan (Ko *et al*. 2023). The previously documented conidial dimensions were 21.5 ± 1.9 × 12.5 ± 0.8 µm, L/W ratio = 1.7 ± 0.2 (Abdollahzadeh *et al*. 2010). The size and L/W ratio of conidia of B2828 were smaller than the reported dimensions, while those of the other three *L. hormozganensis* isolates were similar to the reported ones. This implies that the size and L/W ratio of conidia might differ among *L. hormozganensis* isolates. All 4 isolates did not produce a pink pigment in PDA cultures at 35℃, which was consistent with the previously reported characteristics (Abdollahzadeh *et al*. 2010).

Phylogenetic analysis revealed that eight isolates did not form a well-supported clade with any other previously identified and described *Lasiodiplodia* species; however, they were close to *L. chiangraiensis*, *L. endophytica*, and *L. ireniensis*. The morphology of *L. endophytica* has not been described, so we could not use morphology to determine if these isolates were similar to this species. The conidial dimensions of *L. chiangraiensis* were reported to be (21–)22–27(–30) × (12–)13–15(17) µm, L/W = 1.9 (Wu *et al*. 2021), while those of *L. iraniensis* were smaller, (15.3–)17–23(–29.7) × 11–14, L/W = 1.6 (Abdollahzadeh *et al*. 2010). *L. chiangraiensis* and these 8 unidentified isolates have conidia of similar sizes, but *L. chiangraiensis* is characterized by conidia remaining hyaline even after maturity (Wu *et al*. 2021), while these isolates have colored conidia after maturity, which is the same as *L. ireniensis*. Further studies are needed to decide their taxonomical position.

In conclusion, the species-specific primers developed in this study could accurately identify *L. theobromae* and *L. pseudotheobromae*, 2 of the most prevalent *Lasiodiplodia* species worldwide. These primers could facilitate monitoring of the occurrence of these 2 species in the field and investigating the species diversity of *Lasiodiplodia*. Using these primers to test the collection of *Lasiodiplodia* isolates from avocado, this study found that *L. thailandica*, *L. hormozganensis*, and some unknown *Lasiodiplodia* species were also associated with avocado in Taiwan. In addition, to our knowledge, this is the first study demonstrating that *L. thailandica* is also a pathogen causing avocado stem-end rot disease.

ACKNOWLEDGEMENTS

We thank the Ministry of Agriculture, Taiwan (R. O. C.) for funding this research and Su-Yu Lai, Hsing-Kuei Chen, Li-Zi Yang, and Jia-Da Tsai for assistance with laboratory and field work.

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Appendix 1. GenBank accession numbers for DNA sequences of *Lasiodiplodia* spp. used in the phylogenetic analyses.

		Genbank accession number[y]		
Species	Isolates[z]	*RPB2*	*TEF1-α*	*TUB2*
L. acacia	CBS 136434*	MT592307	*MT592133*	MT592613
L. aquilariae	CGMCC 3.18471*	KY848562	*KY848600*	N/A
L. avicenniae	CMW 41467*	KU587878	*KP860680*	KP860758
L. avicenniae	LAS158	KU587879	KU587946	KU587867
L. bruguierae	CMW41470*	KU587875	*KP860677*	KP860755
L. bruguierae	CMW41614	KU587877	KP860679	KP860757
L. chiangraiensis	MFLUCC 21-0003*	N/A	MW815630	MW815628
L. chiangraiensis	GZCC 21-0003	N/A	MW815629	MW815627
L. chiangraiensis	CDZM 081	OM243836	OM243808	OM228624
L. chonburiensis	MFLUCC 16-0376*	N/A	*MH412773*	MH412742
L. chonburiensis	PSU-LST001	N/A	LC762199	LC762205
L. cinnamomi	CFCC51997*	MH236801	*MH236799*	MH236797
L. citricola	CBS 124707*	KU696351	*GU945340*	KU887505
L. citricola	CBS 124706	KU696350	GU945339	KU887504
L. crassispora	CBS 118741*	KU696353	*DQ103557*	KU887506
L. crassispora (= *L. pyriformis*)	CBS 121770	KU696378	EU101352	KU887527
L. endophytica	MFLUCC 18-1121*	N/A	*MK584572*	MK550606
L. euphorbiaciecola	CMM3609*	N/A	*KF226689*	KF254926
L. euphorbiaciecola	CMW36231	KU696347	KU887063	KU887494
L. gilanensis	IRAN1523C*	KP872462	*GU945342*	KU887511
L. gilanensis	CBS 124705	KU696356	GU945341	KU887510
L. gonubiensis	CBS 115812*	KU696359	*DQ458877*	DQ458860
L. gonubiensis	CMW14078	KU696358	DQ103567	EU673126
L. gravistriata	CMM 4564*	N/A	*KT250950*	N/A
L. hormozganensis	CBS 124709*	KU696361	*GU945343*	KU887515
L. hormozganensis	CBS 124708	KU696360	GU945344	KU887514
L. hormozganensis	MUCC 2724	LC567804	LC567744	LC567774
L. hormozganensis	MUCC 2734	LC567803	LC567743	LC567773
L. hormozganensis	TZS8-1	N/A	OR552338	OR551948
L. hormozganensis	**B2828**	**PP216064**	**PP216045**	**PP378135**
L. hormozganensis	**B2831**	**PQ846691**	**PQ846698**	**PQ846703**
L. hormozganensis	**B2847**	**PQ846692**	**PQ846699**	**PQ846704**
L. hormozganensis	**B2858**	**PQ846693**	**PQ846700**	**PQ846705**
L iraniensis	CBS124710*	KU696363	*GU945336*	KU887516
L. iraniensis	CBS124711	KU696362	GU945335	KU887517
L. iraniensis (= *L. jatrophicola*)	CMW36237	KU696348	KU886998	KU887499
L. iraniensis (= *L. jatrophicola*)	CMW36239	KU696349	KU887000	KU887501
L. lignicola	CBS 134112*	KU696364	*KU887003*	KT852958
L. lignicola	CMW54165	MT920465	MT920442	N/A

Appendix 1. GenBank accession numbers for DNA sequences of *Lasiodiplodia* spp. used in the phylogenetic analyses. (continued)

Species	Isolates[z]	Genbank accession number[y]		
		RPB2	*TEF1-α*	*TUB2*
L. macrospora	CMM 3833*	N/A	*KF226718*	KF254941
L. mahajangana	CMW27801*	KU696365	*FJ900641*	KU887518
L. mahajangana (= *L. exigua*)	CBS137785	KU696355	KJ638336	KU887509
L. margaritacea	CBS122519*	KU696367	*EU144065*	KU887520
L. mediterranea	CBS137783*	KU696368	*KJ638331*	KU887521
L. mediterranea	CBS137784	KU696369	KJ638330	KU887522
L. microcondia	CGMCC 3.18485	KY848561	*KY848614*	N/A
L. parva	CBS 456.78*	KU696372	*EF622063*	KU887523
L. parva	CBS 494.78	KU696373	EF622064	EU673114
L. plurivora	CBS 120832*	KU696374	*EF445395*	KU887524
L. pontae	CMM 1277*	N/A	*KT151791*	KT151797
L. pseudotheobromae	CBS 116459*	KU696376	*EF622057*	EU673111
L. pseudotheobromae	ADN23	**PP396034**	*MN921242*	MT095042
L. pseudotheobromae	AFL8	**PP396035**	*MT086519*	MT095036
L. pseudotheobromae	AZC12	**PP396036**	*MN921241*	MT095041
L. pseudotheobromae	AZC38	**PP396037**	*MN921243*	MT095043
L. pseudotheobromae	**B2852**	N/A	**PV107514**	**PV107500**
L. pseudotheobromae	**B2854**	**PP216065**	**PP216046**	**PP378136**
L. pseudotheobromae	**B2855**	N/A	**PV107515**	**PV107501**
L. pseudotheobromae	**B2857**	N/A	**PV107516**	**PV107502**
L. pseudotheobromae	**B2864**	N/A	**PV107517**	**PV107503**
L. pseudotheobromae	**B2866**	N/A	**PV107518**	**PV107504**
L. pseudotheobromae	**B2871**	N/A	**PV107519**	**PV107505**
L. pseudotheobromae	**B2876**	N/A	**PV107520**	**PV107506**
L. pseudotheobromae	**B2880**	N/A	**PV107521**	**PV107507**
L. pseudotheobromae	**B2885**	PP216066	**PP216047**	**PP378137**
L. pseudotheobromae	**B2888**	N/A	**PV107522**	**PV107508**
L. pseudotheobromae	**B2894**	N/A	**PV107523**	**PV107509**
L. pseudotheobromae	**B2895**	N/A	**PV107524**	**PV107510**
L. pseudotheobromae	**B2942**	N/A	**PV107525**	**PV107511**
L. pseudotheobromae	**B2944**	N/A	**PV107526**	**PV107512**
L. pseudotheobromae	**B2946**	N/A	**PV107527**	**PV107513**
L. rubropurpurea	WAC 12535*	KP872485	*DQ103571*	EU673136
L. rubropurpurea	WAC 12536	KP872486	DQ103572	KU887530
L. subglobosa	CMM3872*	N/A	*KF226721*	KF254942
L. syzygii	MFLUCC 19-0257*	N/A	*MW016943*	MW014331
L. thailandica	**ASS65**	**PP216067**	**PP216048**	**PP378138**
L. thailandica	MUCC2738	LC567810	LC567750	LC567780
L. thailandica	BJFU DZP160119-9	KY676791	KY676797	KY676794

Appendix 1. GenBank accession numbers for DNA sequences of *Lasiodiplodia* spp. used in the phylogenetic analyses. (continued)

		Genbank accession number[y]		
Species	Isolates[z]	*RPB2*	*TEF1-α*	*TUB2*
L. thailandica	BJFU DZP160123-13	KY751298	KY751304	KY751301
L. thailandica (= *L. hyaline*)	CGMCC 3.17975*	KX499955	*KX499917*	KX499992
L. thailandica (= *L. hyalina*)	CGMCC 3.18383	KY751296	KY751302	KY751299
L. thailandica	**B2844**	**PP216068**	**PP216049**	**PP378139**
L. thailandica	**B2848**	**PP216069**	**PP216050**	**PP378140**
L. thailandica	**B2870**	**PP216070**	**PP216051**	**PP378141**
L. thailandica	**B2913**	**PP216071**	**PP216052**	**PP378142**
L. thailandica	**B2932**	**PP216073**	**PP216054**	**PP378144**
L. thailandica	**B2941**	**PP216074**	**PP216055**	**PP378145**
Lasiodiplodia sp.	**AZP74**	**PP216075**	**PP216056**	**PP378146**
Lasiodiplodia sp.	**B2850**	**PQ846694**	**PQ846701**	**PQ846706**
Lasiodiplodia sp.	**B2881**	**PQ846695**	**PQ846702**	**PQ846707**
Lasiodiplodia sp.	**B2903**	**PP216076**	**PP216057**	**PP378147**
Lasiodiplodia sp.	**B2915**	**PP216077**	**PP216058**	**PP378148**
Lasiodiplodia sp.	**B2917**	**PP216078**	**PP216059**	**PP378149**
Lasiodiplodia sp.	**B2928**	**PP216079**	**PP216060**	**PP378150**
Lasiodiplodia sp.	**B2930**	**PP216080**	**PP216061**	**PP378151**
L. theobromae	ACY4	**PP396038**	*MN921239*	MT095039
L. theobromae	ADN21	**PP396039**	*MN921237*	MT095037
L. theobromae	AFL1	**PP396040**	*MT086516*	MT095033
L. theobromae	AZC42	**PP396041**	*MN921240*	MT095040
L. theobromae	AZP58	**PP396042**	*MT086514*	MT095031
L. theobromae	AZP69	**PP396043**	*MT086515*	MT095032
L. theobromae	**B2825**	N/A	**PV167144**	**PV134345**
L. theobromae	**B2827**	N/A	**PV167145**	**PV134346**
L. theobromae	**B2843**	N/A	**PV167146**	**PV134347**
L. theobromae	**B2849**	N/A	**PV167147**	**PV134348**
L. theobromae	**B2862**	N/A	**PV167148**	**PV134349**
L. theobromae	**B2863**	N/A	**PV167149**	**PV134350**
L. theobromae	**B2865**	N/A	**PV167150**	**PV134351**
L. theobromae	**B2873**	N/A	**PV167151**	**PV134352**
L. theobromae	**B2875**	N/A	**PV167152**	**PV134353**
L. theobromae	**B2877**	N/A	**PV167153**	**PV134354**
L. theobromae	**B2886**	N/A	**PV167154**	**PV134355**
L. theobromae	**B2887**	N/A	**PV167155**	**PV134356**
L. theobromae	**B2891**	**PP216081**	**PP216062**	**PP378152**
L. theobromae	**B2900**	**PP216082**	**PP216063**	**PP378153**
L. theobromae	**B2926**	N/A	**PV167156**	**PV134357**
L. theobromae	**B2937**	N/A	**PV167157**	**PV134358**

Appendix 1. GenBank accession numbers for DNA sequences of *Lasiodiplodia* spp. used in the phylogenetic analyses. (continued)

Species	Isolates[z]	Genbank accession number[y]		
		RPB2	*TEF1-α*	*TUB2*
L. theobromae	**B2940**	N/A	**PV167158**	**PV134359**
L. theobromae	**B2943**	N/A	**PV167159**	**PV134360**
L. theobromae	**B2953**	N/A	**PV167160**	**PV134361**
L. theobromae	CBS 164.96*	KU696383	*AY640258*	KU887532
L. theobromae	CBS 111530	KU696382	*EF622054*	KU887531
L. tropica	CGMCC 3.18477*	KY848574	*KY848616*	KY848540
L. venezuelensis	CBS 118739*	KU696384	*DQ103568*	KU887533
L. viticola	CBS 128313*	KU696385	*HQ288269*	HQ288306
L. vitis	CBS 124060*	KX463994	KX464642	KX464917
Diplodia seriata	CBS 112555	KX463962	AY573220	DQ458856

[z] Isolates in bold were obtained in this study; isolates with asterisks are ex-type cultures.

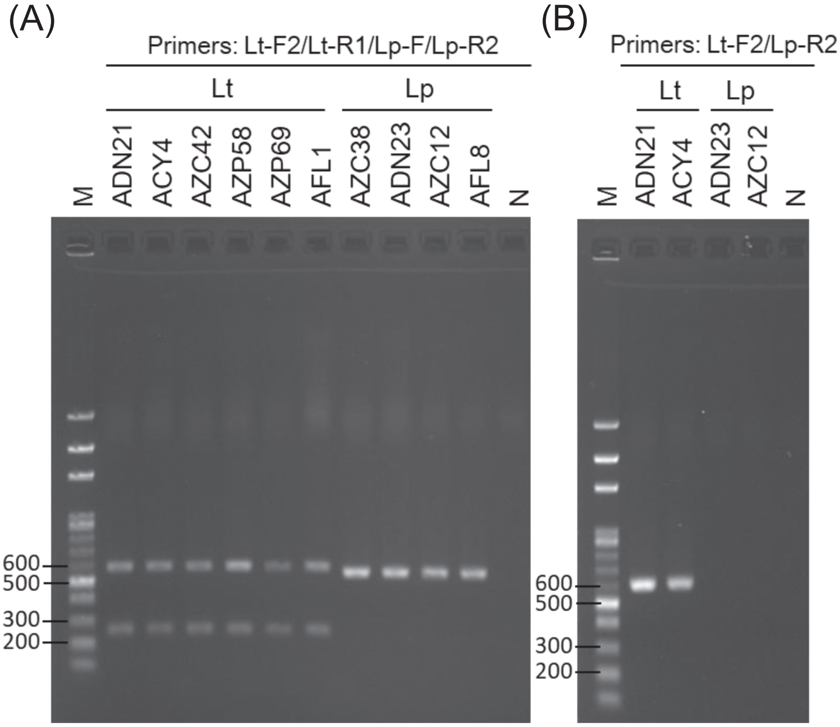
[y] ITS: internal transcribed spacer regions 1 and 2, including the 5.8S ribosomal RNA gene; *RPB2*: RNA polymerase II largest subunit; *TEF1-α*: translation elongation factor 1-α; *TUB2*: β-tubulin; accession numbers in bold indicate sequences obtained in this study; accession numbers in italics indicate sequences used for the design of primers.

Appendix 2. The isolates of *Lasiodiplodia* spp. used in the tests of species-specific primers.

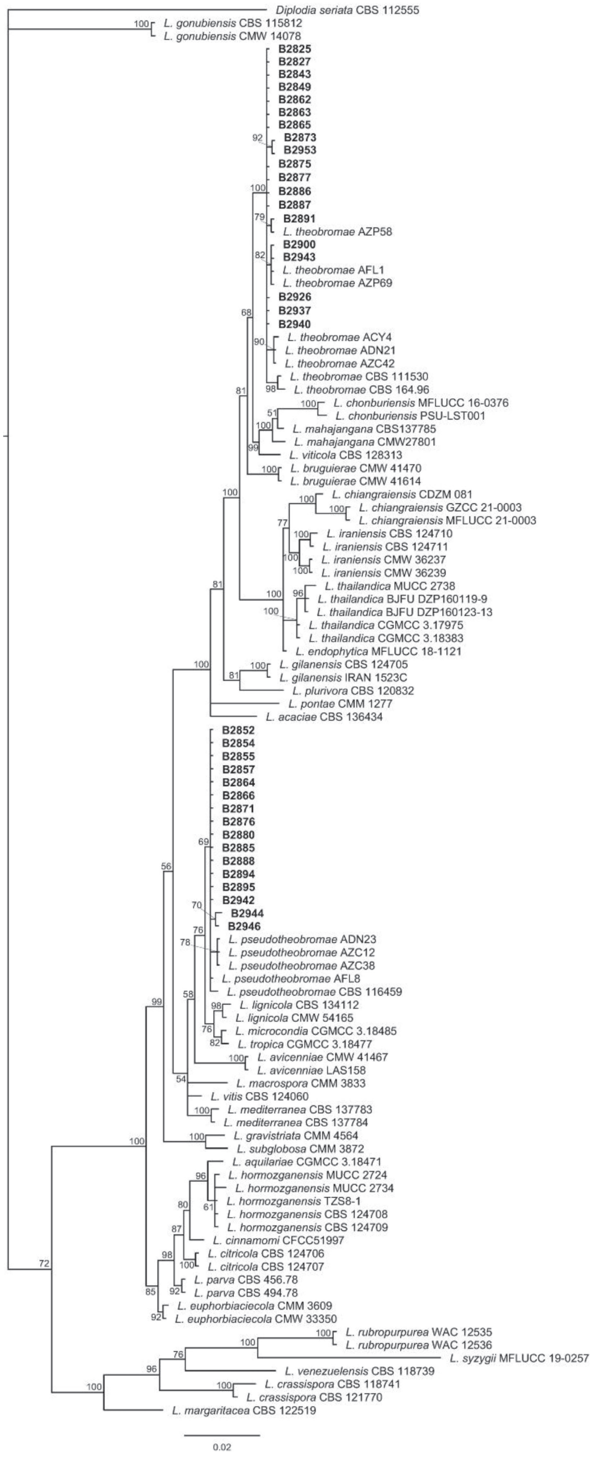
Species	Isolate	Tissue type	Location	Collection date
L. pseudotheobromae	ADN23	branch	Danei, Tainan	2019/1/22
	AFL8	branch	Fanlu, Chiayi	2019/9/30
	AZC12	branch	Zhuqi, Chiayi	2018/9/21
	AZC38	branch	Zhuqi, Chiayi	2019/4/2
	B2852	fruit	Shanshang, Tainan	2020/8/13
	B2854	fruit	Shanshang, Tainan	2020/8/18
	B2855	fruit	Zhuqi, Chiayi	2020/8/24
	B2857	fruit	Zhuqi, Chiayi	2020/8/24
	B2864	fruit	Zhuqi, Chiayi	2020/8/24
	B2866	fruit	Shanshang, Tainan	2020/8/18
	B2871	fruit	Zhuqi, Chiayi	2020/8/24
	B2876	fruit	Zhuqi, Chiayi	2020/8/24
	B2880	fruit	Zhuqi, Chiayi	2020/8/24
	B2885	fruit	Zhuqi, Chiayi	2020/8/24
	B2888	fruit	Shanshang, Tainan	2020/8/18
	B2894	fruit	Zhuqi, Chiayi	2020/8/24
	B2895	fruit	Zhuqi, Chiayi	2020/8/24
	B2942	fruit	Zhuqi, Chiayi	2020/12/3
	B2944	fruit	Zhuqi, Chiayi	2020/12/3
	B2946	fruit	Zhuqi, Chiayi	2020/12/3
L. theobromae	ADN21	branch	Danei, Tainan	2019/1/22
	ACY4	branch	Chiayi City	2019/5/10
	AFL1	branch	Fanlu, Chiayi	2019/9/30
	AZC42	branch	Zhuqi, Chiayi	2019/4/2
	AZP58	branch	Zhongpu, Chiayi	2019/11/1
	AZP69	branch	Zhongpu, Chiayi	2019/11/27
	B2825	fruit	Zhuqi, Chiayi	2020/7/21
	B2827	fruit	Zhuqi, Chiayi	2020/7/31
	B2843	fruit	Zhuqi, Chiayi	2020/8/10
	B2849	fruit	Zhuqi, Chiayi	2020/8/10
	B2862	fruit	Zhuqi, Chiayi	2020/8/24
	B2863	fruit	Shanshang, Tainan	2020/8/21
	B2865	fruit	Zhuqi, Chiayi	2020/8/24
	B2873	fruit	Zhuqi, Chiayi	2020/8/24
	B2875	fruit	Zhuqi, Chiayi	2020/8/24
	B2877	fruit	Zhuqi, Chiayi	2020/8/24
	B2886	fruit	Zhuqi, Chiayi	2020/8/24
	B2887	fruit	Zhuqi, Chiayi	2020/8/24
	B2891	fruit	Shanshang, Tainan	2020/8/23
	B2900	fruit	Zhuqi, Chiayi	2020/8/24
	B2926	fruit	Zhuqi, Chiayi	2020/11/12

Appendix 2. The isolates of *Lasiodiplodia* spp. used in the tests of species-specific primers. (continued)

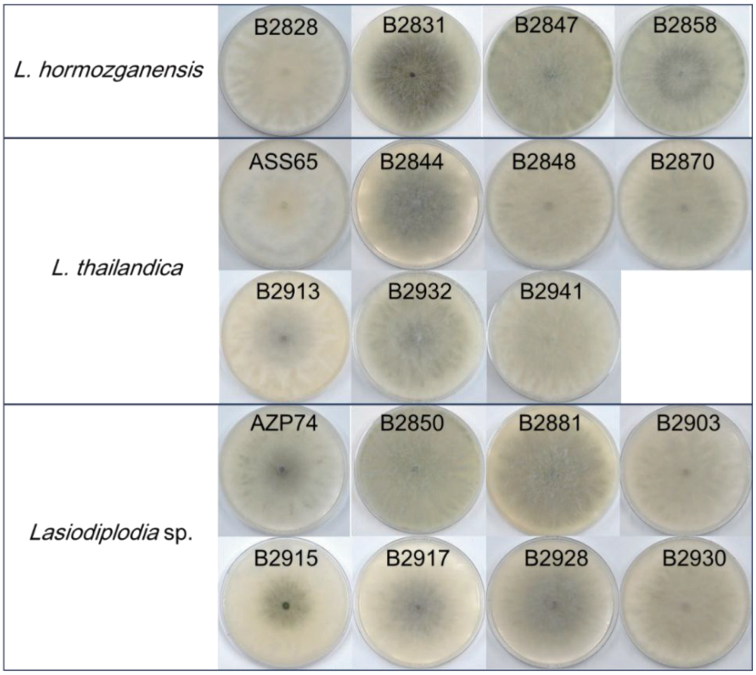
Species	Isolate	Tissue type	Location	Collection date
	B2937	fruit	Zhuqi, Chiayi	2020/12/3
	B2940	fruit	Zhuqi, Chiayi	2020/12/3
	B2943	fruit	Zhuqi, Chiayi	2020/12/3
	B2953	fruit	Zhuqi, Chiayi	2020/12/3
L. thailandica	B2844	fruit	Zhuqi, Chiayi	2020/8/17
	B2848	fruit	Shanshang, Tainan	2020/8/14
	B2870	pedicel	Zhuqi, Chiayi	2020/8/31
	B2913	fruit	Zhuqi, Chiayi	2020/11/12
	B2932	pedicel	Zhuqi, Chiayi	2020/12/1
	B2941	fruit	Zhuqi, Chiayi	2020/11/30
	ASS65	branch	Shanshang, Tainan	2020/9/9
L. hormozganensis	B2828	fruit	Zhuqi, Chiayi	2020/8/7
	B2831	fruit	Zhuqi, Chiayi	2020/7/31
	B2847	fruit	Zhuqi, Chiayi	2020/8/10
	B2858	fruit	Zhuqi, Chiayi	2020/8/24
Lasiodiplodia sp.	AZP74	branch	Zhongpu, Chiayi	2019/12/19
	B2850	fruit	Shanshang, Tainan	2020/8/7
	B2881	fruit	Zhuqi, Chiayi	2020/8/24
	B2903	fruit	Zhuqi, Chiayi	2020/8/24
	B2915	fruit	Zhuqi, Chiayi	2020/11/12
	B2917	fruit	Zhuqi, Chiayi	2020/11/12
	B2928	pedicel	Zhuqi, Chiayi	2020/12/1
	B2930	pedicel	Zhuqi, Chiayi	2020/12/1



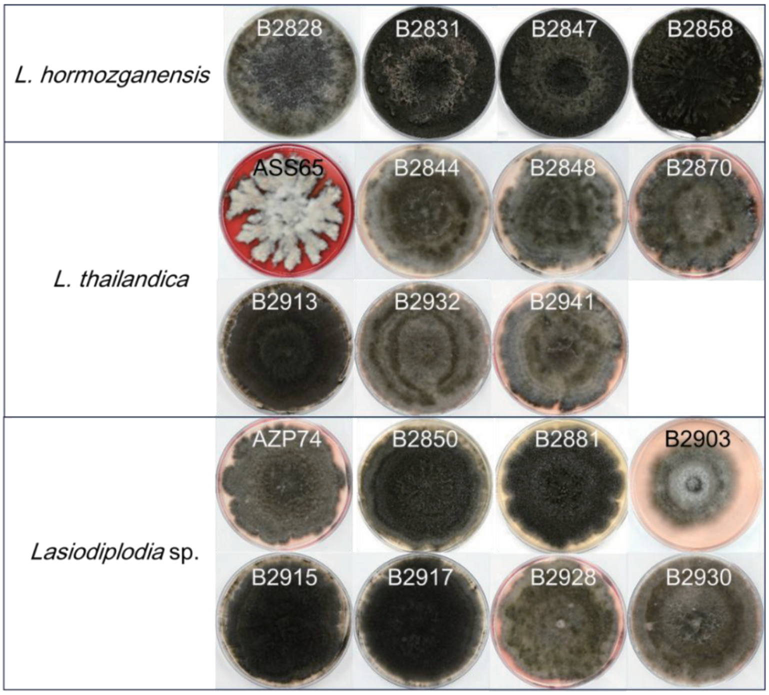
Appendix 3. The results of multiplex PCR with the two species-specific primers pairs (A) Lt-F2/Lt-R1 and Lp-F/Lp-R2 and PCR with (B) Lt-F2/Lp-R2. M: 100-bp DNA ladder (Protech Technology Enterprise, Taipei, Taiwan). N: negative control.



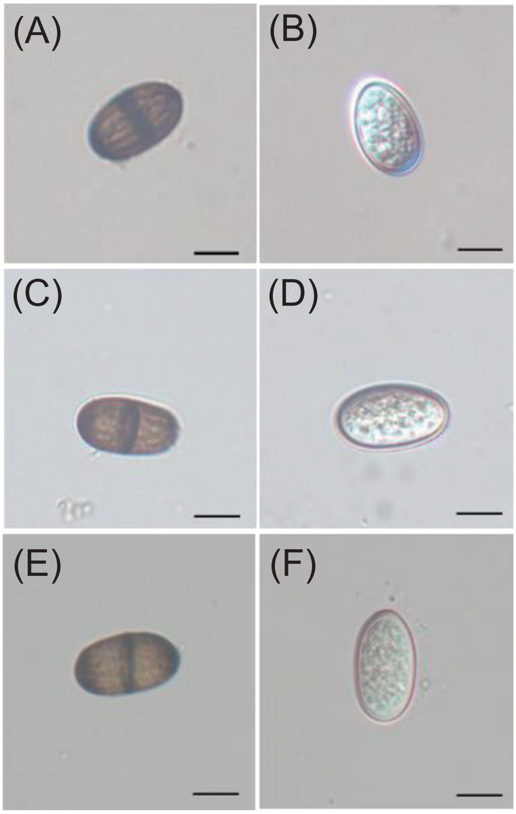
Appendix 4. Bayesian phylogenetic tree of 108 *Lasiodiplodia* isolates from avocado in Taiwan and from the GenBank database. The phylogenetic tree was built using concatenated sequences of partial *TEF1-α* and *TUB2* gene regions. The Bayesian posterior probabilities are indicated next to the nodes. The tree is rooted with *Diplodia seriata* CBS 112555. Isolates from this study are emphasized in bold.



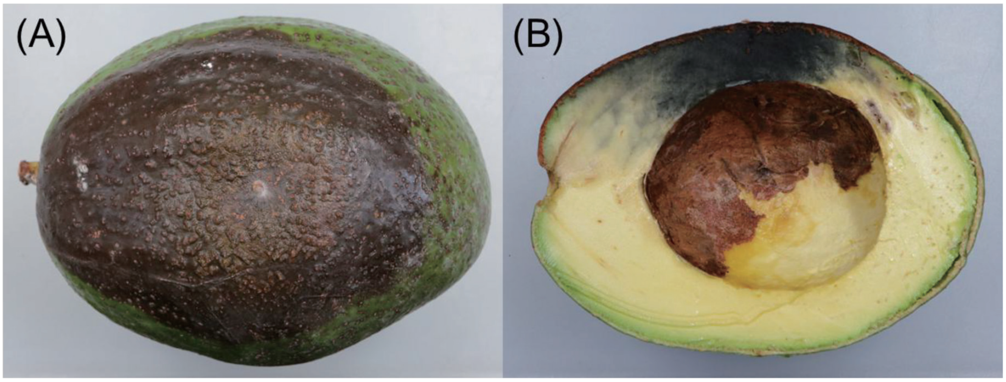
Appendix 5. Colony morphology of the isolates of *Lasiodiplodia hormozganensis*, *L. thailandica*, and unidentified *Lasiodiplodia* isolates cultivated on potato dextrose agar after 3 d at 25℃.



Appendix 6. Colony morphology of the isolates of *Lasiodiplodia hormozganensis*, *L. thailandica*, and unidentified *Lasiodiplodia* isolates cultivated on potato dextrose agar after 14 d at 35℃.



Appendix 7. Conidial morphology of *Lasiodiplodia hormozganensis*, *L. thailandica*, and an unidentified *Lasiodiplodia* isolate. (A) Mature conidia and (B) immature conidium of *L. hormozganensis* B2828. (C) Mature conidium and (D) immature conidium of *L. thailandica* B2848. (E) Mature conidium and (F) immature conidium of *Lasiodiplodia* sp. B2903. Scale bars: 10 μm.



Appendix 8. (A) External lesions and (B) internal discoloration that developed 7 d after artificial inoculation with *Lasiodiplodia thailandica* B2848 on 'Choquette' avocado fruits.

臺灣酪梨上之 *Lasiodiplodia* 屬病原菌之新穎分子鑑定技術

梁鈺平 [1,*]　倪蕙芳 [2]

摘要

梁鈺平、倪蕙芳。2025。臺灣酪梨上之 *Lasiodiplodia* 屬病原菌之新穎分子鑑定技術。台灣農業研究 74(4):459–484。

Lasiodiplodia theobromae 與 *L. pseudotheobromae* 為廣泛分布於熱帶與亞熱帶之植物病原菌，造成許多作物病害，但兩者不易透過傳統型態學方法鑑定與區分。本研究根據 *Lasiodiplodia* 屬物種間之轉譯延長因子 1-α (translational elongation factor 1-α; *TEF1-α*) 序列之差異性，開發了 2 對物種專一性引子對 (Lt-F2/Lt-R1 與 Lp-F/Lp-R2)。以基因體 DNA 進行聚合酶連鎖反應，結果顯示 Lt-F2/Lt-R1 與 Lp-F/Lp-R2 分別對 *L. theobromae* 與 *L. pseudotheobromae* 具有專一性，對於非標的物種則均無擴增反應。使用此二引子增幅分離自臺灣酪梨 (*Persea americana*) 的 54 株 *Lasiodiplodia* 菌株之 DNA，成功自其中鑑定出屬於 *L. theobromae* 與 *L. pseudotheobromae* 的菌株，然有些菌株則無法被以上二對引子增幅，顯示臺灣酪梨上存在其他未知的 *Lasiodiplodia* spp. 病原。進一步以 *TEF1-α*、β 微管蛋白 2 (β-tubulin 2; *TUB2*) 及 RNA 聚合酶 II (RNA polymerase subunit II; *RPB2*) 之基因序列進行以上未知菌株之親緣分析，結果顯示另有 *L. thailandica*、*L. hormozganensis* 及目前尚無法以此親緣關係鑑定的 *Lasiodiplodia* 物種存在於臺灣之罹病酪梨果實、枝條及果梗，經病原性試驗後顯示以上三者對酪梨果實均有病原性。本研究首次報導 *L. thailandica* 為酪梨之病原菌，且本研究開發之專一性引子將可於未來作為鑑別常見病原 *L. theobromae* 與 *L. pseudotheobromae* 的有效工具，有助於研擬正確防治策略。

關鍵詞：*Lasiodiplodia hormozganensis*、*Lasiodiplodia thailandica*、*Lasiodiplodia theobromae*、*Lasiodiplodia pseudotheobromae*、專一性引子。

投稿日期：2025 年 6 月 6 日；接受日期：2025 年 8 月 25 日。

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台灣農業研究 (J. Taiwan Agric. Res.) 74(4):485–495 (2025)
https://doi.org/10.6156/JTAR.202512_74(4).0007

研究報告

由 *Corynespora cassiicola* 引起之沙漠玫瑰葉斑病

黃巧雯 [1,*]　曾淑瓊 [2]　黃晉興 [3]　蔡志濃 [4]

摘要

黃巧雯、曾淑瓊、黃晉興、蔡志濃。2025。由 *Corynespora cassiicola* 引起之沙漠玫瑰葉斑病。台灣農業研究 74(4):485–495。

2022 年 7 月中旬於彰化縣永靖鄉某一處園區，發現沙漠玫瑰葉片上產生褐色圓斑與不規則形病斑，嚴重時造成罹病葉片黃化與落葉。本研究自罹病葉片分離到 1 種真菌，培養於馬鈴薯葡萄糖瓊脂培養基 (potato dextrose agar; PDA) 培養基上，菌落為灰白色至灰色，培養基背面呈棕色至黑色。本菌之分生孢子呈棍棒狀或圓柱狀，直或略彎，淡褐色，具有多個隔膜，頂端鈍圓。依據本病害之病徵表現、病原菌形態特徵以及分子序列鑑定結果，將此菌鑑定為 *Corynespora cassiicola*。經人工接種試驗確認 *C. cassiicola* 對沙漠玫瑰葉片具有病原性，完成柯霍氏法則，證明此菌可為害沙漠玫瑰葉片造成葉斑病。本菌最適菌絲生長溫度為 24−32℃，最適孢子發芽溫度為 20−28℃。測試殺菌劑對其菌絲生長與孢子發芽影響，結果顯示賽普護汰寧、百克列及快得寧可有效抑制本菌菌絲生長，而快得寧、鋅錳乃浦及賽普護汰寧可顯著降低此病原菌之孢子發芽。以上本研究報導沙漠玫瑰葉斑病之病徵表現、病原形態、生長適溫及藥劑敏感性等資料，可作為將來病害鑑定與防治時之參考。本文為臺灣首次紀錄由 *C. cassiicola* 引起的沙漠玫瑰葉斑病報告。

關鍵詞：沙漠玫瑰、葉斑病、葉斑病菌、藥劑篩選。

前言

沙漠玫瑰 (desert rose, impala lily) 又稱天寶花、矮雞蛋花、虎刺花、富貴花、沙紅姬花及亞當花等，學名為 *Adenium obesum* (Forssk.) Roem. & Schult.，屬夾竹桃科 (Apocynaceae)、沙漠玫瑰屬 (*Adenium*) 多年生肉質性灌木，自然分布於阿拉伯半島 (Arabian Peninsula) 至東北非 (Northeastern Africa) 及東非地區 (Eastern Africa) (Colombo *et al*. 2018)，並於 1968 年引進臺灣種植。該灌木花卉原產於乾旱近沙漠地區，其花色鮮紅似玫瑰，因而得名『沙漠玫瑰』。沙漠玫瑰因其耐旱性佳、喜好高溫通風、最適生長溫度為 20–35℃，以及日照與光線充足等生長環境，臺灣中南部的氣候條件相當契合其生長需求。沙漠玫瑰是一種極具觀賞價值的植物，隨著品種日益多樣、花色多元鮮豔，其市場經濟價值日漸提升 (Colombo *et al*. 2018; Lin *et al*. 2020)。

臺灣氣候炎熱潮濕，有利於病蟲害的孳生與傳播，導致作物病蟲害種類繁多，根據《臺灣植物病害名彙》與相關研究報告指出，危害沙漠玫瑰的病原紀錄僅 3 種病害，由 *Phytophthora nicotianae* 引起的疫病、由 *Cucumber mosaic virus* (CMV) 引起的胡瓜嵌紋病毒以及由 *Plumeria mosaic virus* (PluMV) 引起的緬梔嵌紋病毒 (Tzean *et al*. 2019; Lin *et al*. 2020)。然而，2022 年 7 月中旬於彰化縣永靖鄉某一處園區發現一未記錄病害，受害葉片呈淡褐色或灰褐圓斑，逐漸擴大為圓形或不規則形病斑，中間

投稿日期：2025 年 5 月 15 日；接受日期：2025 年 8 月 21 日。
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有時呈現明顯同心輪紋，病斑周緣有時產生淡淡黃暈，嚴重時會造成罹病葉片提早黃化與落葉。經病原分離、鏡檢病原形態及依柯霍氏法則 (Koch's postulates) 進行病原性測定後，確認為由 *Corynespora cassiicola* 引起之沙漠玫瑰葉斑病。在臺灣，已知 *C. cassiicola* 為胡瓜褐斑病、木瓜褐斑病、番茄斑點病及羅勒枝枯病之病原菌 (Tzean *et al*. 2019; Tang *et al*. 2021)，本研究顯示此病原亦為沙漠玫瑰葉斑病之病原菌。

由於在臺灣未見由 *C. cassiicola* 引起之沙漠玫瑰葉斑病研究報告，因此本研究除對沙漠玫瑰葉斑病菌的形態、生理特性及病原性測定進行研究探討與瞭解外，另就現已推薦防治觀賞花卉或花木之真菌性病害之藥劑進行本病害之室內防治藥劑篩選，期望藉此能篩選出有效藥劑以提供此病害防治上之參考。

材料與方法

病原菌分離與保存

本研究測試之菌株來自彰化縣永靖鄉某一處園區，由感染葉斑病的沙漠玫瑰葉片上分離而得。分離的方式為將罹葉斑病之葉片取下，並以解剖刀切下病斑之病健部組織，以 75% 酒精表面消毒後，置於以 2% (w/v) 水瓊脂 (water agar; WA) 平板上，待病組織長出菌絲後以移植針切取菌絲尖端移到馬鈴薯葡萄糖瓊脂培養基 (potato dextrose agar; PDA) 培養基上培養。選取分離所得之菌株 Cory-001 與 Cory-002 分別置於 PDA 上培養 5 d 後，再挑取菌絲塊移植於含滅菌沙漠玫瑰枝條之 2% (w/v) WA 平板上，待其產孢後進行單孢分離，單孢分離株移至 2% (w/v) WA 培養基上培養，並將菌絲塊置入裝無菌水之保存管中，於 24℃定溫室保存待用。

病原菌形態與分子生物鑑定

將 Cory-001 與 Cory-002 菌株培養於 PDA 培養基上，觀察菌落等外觀形態；並將分離株培養於表面置有沙漠玫瑰枝條之 2% WA 培養基上，以 24℃定溫培養約 7 d，待其產孢後，以滅菌之針頭挑取分生孢子置於載玻片上，以光學顯微鏡 (Differential Interference Contrast, DIC, LEICA DM 2500, Leica Microsystems GmbH, Wetzlar, Germany) 進行孢子形態觀察，並以 NIS-Elements BR 3.0 軟體 (Nikon, Tokyo, Japan) 測量 50 個以上分生孢子之長度與寬度。分子鑑定方面則是將分離菌株單孢培養於 PDA 培養基平板上 7 d 後，委託源資國際生物科技股份有限公司 (Tri-I Biotech Inc., Taichung, Taiwan) 進行聚合酶鏈鎖反應 (polymerase chain reaction; PCR) 與 DNA (deoxyribonucleic acid) 定序，使用之引子對為 ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) 與 ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) (White *et al*. 1990)，擴增核醣體內轉錄區間 (ribosomal internal transcribed spacer (ITS)) 序列與分離株之 DNA 序列直接定序，定序後序列在 NCBI (National Center for Biotechnology Information) 資料庫中進行序列分析比對，並將該序列登錄於 NCBI 基因資料庫。

接種源配製與病原性測定

將 Cory-001 分離株培養於含滅菌後沙漠玫瑰枝條之 2% (w/v) WA 平板上，置於 24℃、12 h 光照下培養約 7 d 後，以無菌水洗下培養皿內之分生孢子，並利用血球計數器 (hemacytometer, Bright-Line, Hausser Scientific, Horsham, PA, USA) 測定孢子濃度，再以無菌水製成孢子懸浮液 (10^6 spores mL^{-1}) 供接種試驗用。購買市售沙漠玫瑰植株，將 *C. cassiicola* 分離株之孢子懸浮液分別進行離體葉片與盆栽葉片之接種試驗。在離體葉片上接種方式如下：將沙漠玫瑰葉片由植株取下後，以滅過菌 3 號蟲針在葉片上製造傷口，將 *C. cassiicola* Cory-001 與 Cory-002 菌株以無菌水配製孢子懸浮液 (10^5 spores mL^{-1})，分別取 20 μL 滴於傷口上，每片葉片於左右兩側各處理 1 點，每個處理 4 片離葉，並以無菌水作為對照組，再將接種後葉片保濕於密封塑膠盒中，分別放置於 25℃黑暗之定溫箱中，逐日觀察病斑進展情形，本實驗重複進行 2 次。另外在盆栽

上葉片接種方式如下：將上述以無菌水配製孢子懸浮液，均勻噴布於盆栽葉片上，接種後盆栽隨即進行套袋保濕 2 d 後去除塑膠袋，盆栽放置於溫室內觀察病勢進展情形，對照組則以無菌水進行接種，每處理 4 重複，本實驗重複進行 2 次。

溫度對病原菌菌絲生長與孢子發芽之影響

將 Cory-001 與 Cory-002 菌株分別移植於 PDA 培養基平板上並置於室溫培養 7 d 後，以口徑 0.6 cm 滅菌過之打孔器切取菌絲邊緣，將菌絲塊置於 PDA 平板中央，分別置於 4、8、12、16、20、24、28、32、36 及 40℃之定溫箱中黑暗培養，於培養後第 9 天測量其菌絲生長之直徑，每個處理 4 重複，本實驗重複進行 2 次。另外，將 Cory-001 與 Cory-002 菌株分別培養於含滅菌後沙漠玫瑰枝條之 WA 上待其產孢後，以無菌水配製孢子懸浮液 (約 10 spores μL^{-1})，再以無菌微量吸管吸取 20 μL 供試菌株之孢子懸浮液，分別置於 4、8、12、16、20、24、28、32、36 及 40℃之定溫箱中，於 24 h 後取出於顯微鏡計算 100 個孢子之發芽情形，每個處理 4 重複，本實驗重複進行 2 次。

藥劑對 *C. cassiicola* 病原菌菌絲生長之影響

將供試菌株 Cory-001 與 Cory-002 移植至 PDA 平板上，於室溫培養 7 d 後，以滅菌過孔徑 0.6 cm 之打孔器切取菌絲塊供試，並利用藥劑平板測試法測定供試藥劑之抑菌效果。所使用 13 種藥劑及種類如下：42.4% 白克列水懸劑 (Boscalid，興農股份有限公司，臺灣臺中市)、10% 保粒黴素 (甲) 可濕性粉劑 (Polyoxin-B，興農股份有限公司，臺灣臺中市)、50% 貝芬替水懸劑 (Carbendazim，富農化學工業股份有限公司，臺灣桃園市)、33.5% 快得寧水懸劑 (Oxine-copper，興農股份有限公司，臺灣臺中市)、40% 甲基多保淨水懸劑 (thiophanate methyl，興農股份有限公司，臺灣臺中市)、22.7% 腈硫醌水懸劑 (Dithianon，興農股份有限公司，臺灣臺中市)、23.6% 百克敏乳劑 (Pyraclostrobin，雅飛有限公司，臺灣臺北市)、62.5% 賽普護汰寧水分散性粒劑 (Cyprodinil + Fludioxonil，先正達股份有限公司，臺灣臺北市)、33% 鋅錳乃浦水懸劑 (Mancozeb，興農股份有限公司，臺灣臺中市)、75% 四氯異苯腈可濕性粉劑 (Chlorothalonil，興農股份有限公司，臺灣臺中市)、18% 賽福寧乳劑 (Triforine，立農化學股份有限公司，臺灣雲林縣)、23.7% 依普同水懸劑 (iprodione，興農股份有限公司，臺灣臺中市) 及 44.2% 克收欣水懸劑 (Kresoxim-methyl，嘉泰企業股份有限公司，臺灣桃園市)，配製成含有效成分濃度為 1、10 及 100 mg a.i. L^{-1} 之 PDA 培養基，另以不添加藥劑之 PDA 平板作為對照。將上述直徑 0.6 cm 的菌絲塊，菌絲面朝下置入直徑 8.5 cm 之含藥的 PDA 培養基平板中央，置於 25℃之定溫箱中黑暗培養，於培養後 14 d 測量其菌絲生長直徑，每處理 4 重複，本試驗重複進行 3 次。試驗結果按下列公式換算藥劑對菌絲之生長抑制率：抑制率 (%) = [(對照組平均生長直徑 − 藥劑處理組平均生長直徑)/對照組平均生長直徑] × 100%。

藥劑對 *C. cassiicola* 分生孢子發芽之影響

以無菌微量吸管吸取 50 μL 上述不同藥劑之溶液，置於 3 凹載玻片之凹槽內，再以無菌微量吸管吸取 1 μL 供試之 Cory-001 與 Cory-002 孢子懸浮液 (200 conidia μL^{-1})，滴於上述含農藥種類：白克列、保粒黴素 (甲)、貝芬替、快得寧、百克敏、賽普護汰寧、鋅錳乃浦及克收欣等 6 種之載玻片凹槽內，並混合均勻，使凹槽內混合液之有效成分濃度為 10 mg a.i. L^{-1}。供試載玻片置於加有 5–10 mL 無菌水之 8.5 cm 塑膠培養皿中，以避免玻片水分蒸散，並將培養皿置於 25℃之定溫箱中，經 24 h 後於顯微鏡下逢機計算 100 個孢子之發芽率，每個處理 4 重複，本試驗重複進行 2 次。以滅菌蒸餾水處理作為對照組。

統計分析

各項處理之試驗資料利用 SAS 9.1 版統計分析軟體先進行變方分析 (analysis of variance variance; ANOVA)，以最小顯著性差異 (least

significant difference; LSD) 測驗在 5% 顯著水準下 ($P = 0.05$)，比較處理間平均值之差異。

結果

沙漠玫瑰葉斑病之病徵

2022 年 7 月中旬於彰化縣永靖鄉某一處園區發現沙漠玫瑰葉片上產生褐色圓形病斑，初期受害葉片呈淡褐色或灰褐圓斑，逐漸擴大為圓形或不規則形病斑，中間有時呈現明顯同心輪紋，病斑周緣有時產生淡淡黃暈，嚴重時會造成罹病葉片黃化與落葉 (圖 1)。

沙漠玫瑰葉斑病菌之培養形態與分子鑑定

本研究自罹病葉片分離到 1 種真菌，培養於 PDA 培養基上，菌落為灰白色至灰色，培養基背面呈棕色至黑色 (圖 2A)。將罹病葉片放置於濕度箱中約 2−4 d 後，於病徵上產生分生孢子梗或分生孢子。本菌之分生孢子為棍棒狀或圓柱狀，直或略彎，淡褐色，具有多個隔膜，頂端鈍圓，大小約為 52.9−118.3 μm × 6.9−10.8 μm，分生孢子以鏈生方式產孢 (圖 2B 與 2C)。為進一步確認菌株鑑定之正確性，將 Cory-001 與 Cory-002 菌株之 ITS 片段序列純化定序後，並登錄於 NCBI 之 GenBank，其序列之 Accession number 為 OR481069 與 OR481070。再利用多重序列比對分析菌株間之序列相同度，發現與 NCBI 上登錄之 *C. cassiicola* 菌株 (Accession No. MW696164) 的 ITS 序列相同度均為 100%。

病原性測定

沙漠玫瑰褐斑病菌分離株以孢子懸浮液 (10^6 spores mL^{-1}) 分別接種於離葉傷口與盆栽葉片。結果顯示，分離株 Cory-001 與 Cory-002 分別以孢子懸浮液接種於以針刺製造傷口處理之離體葉片上，於接種 2 d 後，葉片上接種處開始產生淡褐色斑點，病斑逐漸往外擴散，於接種 3 d 後，葉片上病斑轉為黑褐色圓形病斑更為明顯 (圖 3A 與 3B)；而在盆栽試驗，*C. cassiicola* Cory-001 以孢子懸浮液直接噴布於葉片上，於接種 5 d 後開始出現淡褐色斑點病斑，於接種 14 d 後葉片上病斑逐漸擴大為圓形或不規則形之淺褐色病斑 (圖 4A 與 4B)，對照組則是以無菌水處理之離體葉片及盆栽葉片上，均未見病斑產生 (圖 3C 與圖 4C)。以後再從罹病組織回分得到菌株與接種病原菌菌株，其形態特徵皆相同，並完柯霍氏

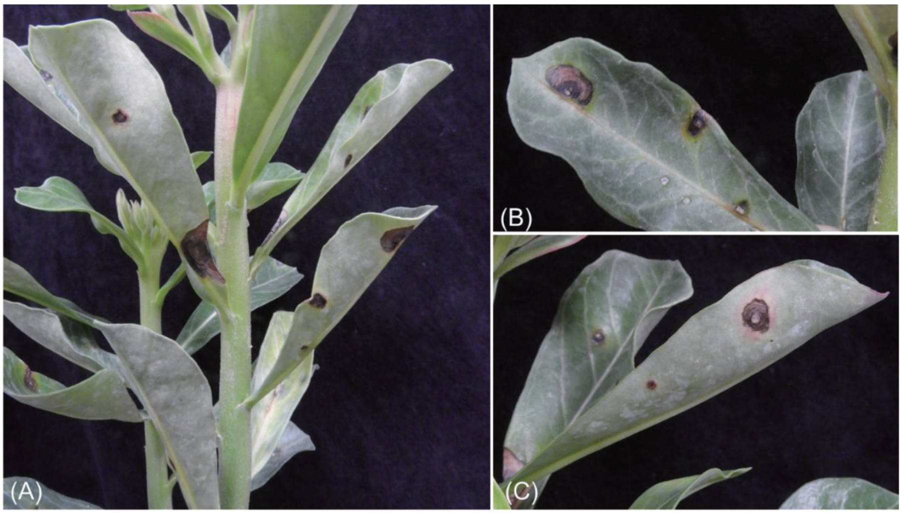

圖 1.　沙漠玫瑰葉斑病葉片上病徵。(A) 於田間自然感染植株葉片上所造成的病徵；(B) 葉面上病徵；(C) 葉背上病徵。

Fig. 1.　(A) Naturally infected leaf spot symptoms of desert rose. Symptoms of dark brown spot were observed on (B) leaf surface and (C) leaf back.

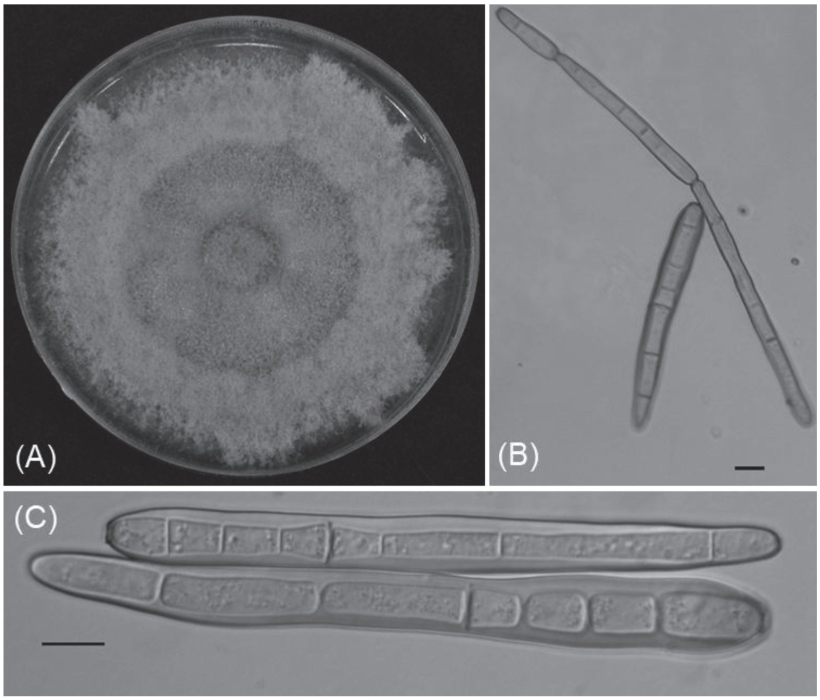

圖 2. 沙漠玫瑰葉斑病菌之菌落與分生孢子形態。(A) 沙漠玫瑰葉斑病菌於 PDA 培養基上於 24℃培養 7 d 後的菌落形態；(B) 分生孢子以鏈生方式產孢；(C) 分生孢子，內具橫格。標尺 = 10 μm。

Fig. 2. Colony and conidial morphology of *Corynespora cassiicola*. (A) Colony of *C. cassiicola* on PDA at 24℃ after cultured for 7 d, (B) conidial chain and (C) conidia with septa. Bars denote 10 μm.

圖 3. 沙漠玫瑰葉斑病病原菌 (*Corynespora cassiicola* Cory-001、Cory-002) 分別接種於沙漠玫瑰離葉葉片上之病徵表現。(A) 以 Cory-001 菌株接種；(B) 以 Cory-002 菌株接種；(C) 以無菌水接種為對照組。

Fig. 3. Dark brown lesion symptoms caused by inoculating (A) *Corynespora cassiicola* Cory-001 and (B) Cory-002 through wounds on detached leaves of desert rose. (C) Inoculation with sterile water as control.

法則 (Koch's postulates)，證實本研究所分離之 *C. cassiicola* 對沙漠玫瑰葉片具有病原性。

溫度對病原菌菌絲生長及孢子發芽之影響

將供試的 2 株葉斑病菌之菌絲塊培養於不同溫度之培養箱中，其生長結果如圖 5 所示，Cory-001 與 Cory-002 分離株於 8–28℃培養時，其菌絲生長隨著溫度升高而增快，Cory-001 菌株於 24–28℃培養時，對菌絲生長最為有利，於培養後 12 d 時其平均生長直徑分別為 8.3 cm 與 8.1 cm；Cory-001 菌株於 32–40℃下生長速率遞減，至 40℃時完全不生長。Cory-002 菌株則在 28℃培養時，對菌絲生長最佳，但至 40℃培養時病原則菌絲則完全不生長。另外，溫度對孢子之發芽影響結果則如圖 6 所示，兩菌株在 16–28℃之發芽率介於 63–88% 之間，就 Cory-001 菌株而言，於 20℃培養時，對孢子發芽最有利溫度，其孢子發芽率為 77.5%；Cory-002 菌株則在 28℃培養時，對孢子發芽為最佳溫度，其孢子發芽率為 88.3%，於 4℃下兩菌株孢子則完全不發芽。

圖 4. (A–B) 沙漠玫瑰葉斑病病原菌 (*Corynespora cassiicola* Cory-001) 接種於沙漠玫瑰葉片上之病徵表現；(C) 以水作為對照組。

Fig. 4. (A–B) Disease symptoms showing on desert rose leaves after inoculation with *Corynespora cassiicola* Cory-001 isolate for 14 d. (C) Inoculation with sterile water as control group.

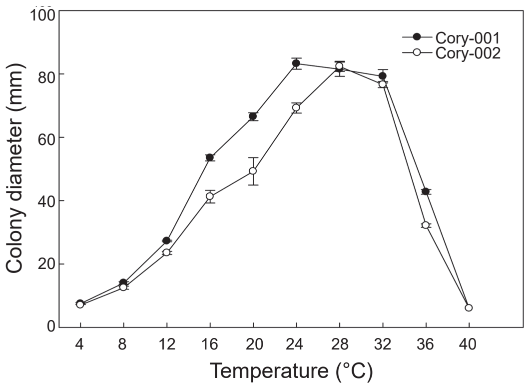

圖 5. 不同溫度對沙漠玫瑰葉斑病菌 *Corynespora cassiicola* Cory-001 與 Cory-002 分離株病原菌菌絲生長之影響 (在 PDA 平板培養 9 d)。

Fig. 5. Effect of temperature on the mycelial growth of *Corynespora cassiicola* Cory-001 and Cory-002 isolates cultured on potato dextrose agar (PDA) plates for 9 d.

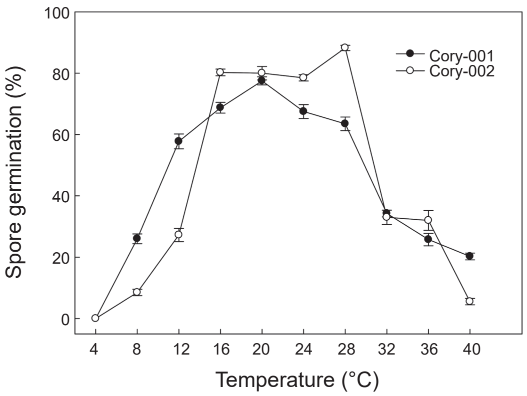

圖 6. 不同溫度對沙漠玫瑰葉斑病菌 *Corynespora cassiicola* Cory-001 與 Cory-002 分離株孢子發芽之影響。

Fig. 6. Effect of temperature on spore germination of *Corynespora cassiicola* Cory-001 and Cory-002 isolates.

藥劑對 *C. cassiicola* 病原菌菌絲生長之影響

將供試藥劑添加於 PDA 平板上，測試各藥劑對 C. cassiicola 病原菌菌絲生長之抑制情形，結果如表 1 所示。賽普護汰寧於 1 mg a.i. L^{-1} 有效成分藥劑濃度下，對 Cory-001 與 Cory-002 分離株之菌絲生長抑制率皆在 45% 以下。若於 10 mg a.i. L^{-1} 有效成分濃度下，依普同、百克敏、快得寧及賽普護汰寧對 Cory-001 菌絲生長之抑制率分別為 63.5%、54.8%、53.5% 及 48.4%，而對 Cory-002 菌絲生長之抑制率分別為 56.5%、34.8%、51.8% 及 68.0%。若有效成分濃度提高至 100 mg a.i. L^{-1} 下，則賽普護汰寧與百克敏對 Cory-001 菌絲生長抑制率分別提高至 94.6% 與 84.0%，而 Cory-002 亦有 76.4% 與 71.4% 的菌絲生長抑制率，與其他不同藥劑處理之結果具有顯著性差異，而保粒黴素、貝芬替、四氯異苯腈及克收欣即使在 100 mg a.i. L^{-1} 有效成分濃度下，對 2 株菌株菌絲生長亦僅不到 20.0% 之生長抑制率。

藥劑對 *C. cassiicola* 病原菌分生孢子發芽之影響

測試藥劑對 *C. cassiicola* 病原菌分生孢子發芽之影響， 結果如表 2 所示。Cory-001 與 Cory-002 分離株於 1 mg a.i. L^{-1} 有效成份藥劑濃度下，快得寧處理其分生孢子發芽抑制率皆為 100%，對於孢子發芽具有極佳的抑制性，其次為鋅錳乃浦處理下，分別仍有 88.8% 與 100% 的抑制率，與其他不同藥劑處理之結果具有顯著性差異。當有效成份濃度提高至 10 mg a.i. L^{-1} 下，快得寧、賽普護汰寧及鋅錳乃浦等 3 種藥劑處理下，其 2 株病原菌之分生孢子發芽之抑制率皆為 90% 以上，而在 10 mg a.i. L^{-1} 有效成分濃度，Cory-001 菌株而言，百克敏、克收欣及百克列處理下分別僅有 22.9%、33.0% 及 52.3% 的發芽率；Cory-002 菌株則仍分別有 67.5%、75.9% 及 51.6% 的發芽率。

討論

C. cassiicola 是一種重要且廣泛分布的真菌病原，屬 Corynesporascaceae 科，不僅是一種常見的壞疽型病原菌 (necrotrophic pathogen)，*C. cassiicola* 還可為腐生菌 (saprotrophs) 或內生菌 (endotrophs) (Kingsland 1986; Collado *et al*. 1999; Suryanarayanan *et al*. 2002; Lee *et al*. 2004; Promputtha *et al*. 2007; Déon *et al*. 2012)，寄主範圍廣泛，可感染熱帶與亞熱帶地區之重要經濟作物、觀賞植物或一些常見的雜草，如黃瓜、木瓜、番茄、南瓜、棉花、大豆、甜椒、豆類、萵苣、羅勒、橡膠樹、繡球花、牽牛花、象草及百合草等，寄主植物高達上百種以上 (Dixon *et al*. 2009; Pereira *et al*. 2019; Tang *et al*. 2021)，所造成之病徵有葉片褐斑與斑點，枝條枯萎，莖、根及塊莖腐爛等，為多種高經濟價值作物生產中的重要病害之一，亦造成人類疾病發生 (Mahgoub 1969; Huang *et al*. 2010)。在臺灣，已知 *C. cassiicola* 為胡瓜褐斑病、木瓜褐斑病、番茄斑點病及羅勒枝枯病之病原菌 (Tzean *et al*. 2019; Tang *et al*. 2021)，本研究顯示亦為沙漠玫瑰葉斑病之病原菌。根據 Dixon *et al*. (2009) 報告指出，*C. cassiicola* 該病原對多種寄主具有致病性 (pathogenicity) 外，其中有些分離株則表現出寄主專一性 (host specificity)，得知此病原菌與寄主關聯性方面具有高度多樣性。因此，本研究所分離的 Cory-001 與 Cory-002 菌株對沙漠玫瑰葉片具有病原性，但對其他作物其致病性與否或是否具有寄主專一性，有待未來進一步釐清。

本研究另測試溫度對 *C. cassiicola* 菌株菌絲生長及孢子發芽之影響，結果得知 24–28℃ 為本菌菌絲最適生長之溫度，而 8℃以下與 40℃之溫度，菌絲幾乎停止生長；在 16–28℃ 無菌水存在 24 h 之情形下，所測試的兩菌株之分生孢子發芽率均可達 60% 以上，顯示臺灣的溫暖氣候下有利於本病原菌之發芽與侵入感染。由文獻指出 (Pernezny *et al*. 2000; MacKenzie *et al*. 2018)，當環境濕度接近 100% 的長時間條件下 (16–44 h)，*C. cassiicola* 分生孢子即可在植物罹病組織上產生，再藉由風吹散或雨水飛濺傳播；當溫度介於 25–35℃，最適合於本菌之侵染，導致病害之發生。另外，*C. cassiicola* 可在感染的寄主植物組織上存活超過 2 年 (Boosalis & Hamilton 1957; MacK-

表 1.　不同藥劑對 *Corynespora cassiicola* Cory-001 與 Cory-002 分離株菌絲生長之影響。

Table 1.　Effect of fungicides on mycelial growth of *Corynespora cassiicola* Cory-001 and Cory-002 isolate by culture plate method.

	Inhibition (%)[z]					
	Cory-001			Cory-002		
Fungicide[y]	1 mg a.i. L^{-1}	10 mg a.i. L^{-1}	100 mg a.i. L^{-1}	1 mg a.i. L^{-1}	10 mg a.i. L^{-1}	100 mg a.i. L^{-1}
42.4% Boscalid (SC)	27.9 b[x]	42.0 c	84.0 b	11.2 bcd	45.4 bc	71.4 ab
50% Polyoxins (SG)	5.9 d	5.6 f	11.4 g	0.9 d	3.6 d	1.4 g
50% Carbendazim (SC)	11.7 cd	15.7 ef	13.9 g	5.5 cd	2.8 d	13.1 fg
33.5% Oxine-copper (SC)	13.0 bcd	53.5 ab	67.3 c	3.6 d	51.8 b	70.8 ab
40% Thiophanate-Methyl (SC)	6.6 d	19.6 de	24.0 ef	2.8 d	0.0 d	8.1 g
22.7% Dithianon (SC)	22.8 bc	16.8 de	30.3 e	2.8 d	2.9 d	28.1 ef
23.6% Pyraclostrobin (EC)	22.4 bc	54.8 ab	54.2 d	12.1 bcd	34.8 c	51.2 cd
62.5% Cyprodinil + Fludioxonil (WG)	45.5 a	48.4 bc	94.6 a	47.7 a	68.0 a	76.4 a
33% Mancozeb (SC)	15.1 bcd	15.7 ef	46.2 d	16.1 bc	9.3 d	38.9 de
75% Chlorothalonil (WP)	9.0 cd	26.9 d	18.3 fg	17.9 b	6.3 d	17.8 fg
75% Triforine (EC)	4.2 d	16.7 de	69.1 c	7.2 bc	7.5 d	57.9 bc
23.7% Iprodione (SC)	13.1 bcd	63.5 a	66.8 c	0.0 d	56.5 b	66.2 abc
44.2% Kresoxim-methyl (SC)	9.9 cd	9.0 ef	16.3 fg	1.8 d	5.7 d	0.0 g
LSD (P = 0.05)	15.1	10.7	10.0	12.3	13.0	18.2

[z] Inhibition (%) = [(Diameter of mycelial growth on PDA without fungicide - diameter of mycelial growth on PDA with fungicide)/diameter of mycelial growth on PDA without fungicide] × 100.

[y] SC: suspension concentrate; EC: emulsifiable concentrate; WG: water-dispersible granules; WP: wettable powder.

[x] Means within a column followed by the same letter are not significantly different at 5% by least significant difference (LSD) test.

表 2. 不同藥劑對 *Corynespora cassiicola* Cory-001 與 Cory-002 分離株孢子發芽之影響。

Table 2. Effect of fungicides on spore germination of *Corynespora cassiicola* Cory-001 and Cory-002 isolate by glass slide method.

	Inhibition (%)[z]			
	Cory-001		Cory-002	
Fungicide[y]	1 mg a.i. L^{-1}	10 mg a.i. L^{-1}	1 mg a.i. L^{-1}	10 mg a.i. L^{-1}
42.4% Boscalid (SC)	33.4 d[x]	52.3 c	22.0 d	51.6 b
33.5% Oxine-copper (SC)	100.0 a	100.0 a	100.0 a	100.0 a
23.6% Pyraclostrobin (EC)	17.2 e	22.9 e	60.5 c	67.5 c
62.5% Cyprodinil + Fludioxonil (WG)	35.7 c	90.4 b	81.6 b	100.0 a
33% Mancozeb (SC)	88.8 b	94.2 ab	100.0 a	100.0 a
44.2% Kresoxim-methyl (SC)	3.3 f	33.0 d	3.4 e	75.9 b
LSD (P = 0.05)	3.5	5.9	4.3	4.0

[z] Inhibition (%) = [(No. of spore germinate on water without fungicide − No. of spore germinate on water with fungicide)/No. of spore germinate on water without fungicide] × 100.
[y] SC: suspension concentrate; EC: emulsifiable concentrate; WG: water-dispersible granules.
[x] Means within a column followed by the same letter are not significantly different at 5% by least significant difference (LSD) test.

enzie *et al*. 2018)，因此田間衛生管理尤為重要，如清除罹病組織、枝條是防治本病害發生之重要防治策略之一。

有關沙漠玫瑰葉斑病的防治，目前在國內並無推薦藥劑，本研究以推薦在觀賞花卉之其他真菌性病害之藥劑，進行沙漠玫瑰葉斑病菌絲生長與孢子發芽之影響測試，試驗結果顯示，對菌絲生長抑制效果最佳的藥劑為賽普護汰寧與百克列，次佳者為快得寧；抑制孢子發芽的藥劑，則以快得寧與鋅錳乃浦效果最佳，賽普護汰寧為次佳者。綜合而論，對孢子發芽與菌絲生長都有良好抑制效果者為快得寧與賽普護汰寧。從室內藥劑篩選結果，上述藥劑可作為防治沙漠玫瑰葉斑病之用藥。值得注意的是，*C. cassiicola* 本病原菌對殺菌劑的抗藥性被列為高度抗藥性風險族群 (Sun *et al*. 2022)，於田間進行藥劑防治時，應輪用不同作用機制的藥劑，且應降低苯咪唑類 (benzimidazoles) 藥劑如免賴得、貝芬替及腐絕等與醌類抑制劑 (QoIs) 藥劑如克收欣、亞托敏及百克敏等使用頻度或謹慎施用，為降低或延緩抗藥性的產生。

由本研究結果顯示沙漠玫瑰葉斑病之病原菌 *C. cassiicola* 適合於臺灣氣候條件下生長與侵染，其感染源可能來自殘存於田間枯葉上產生之病原菌分生孢子，藉由風雨途徑進行侵染。因此，田間衛生尤為重要一環，清園可降低田間病原菌密度，此外適當修剪枝條以促進通風亦有助於降低病害發生機率。藥劑防治上，由本研究所篩選的賽普護汰寧、百克列及快得寧可有效抑制本菌菌絲生長，而快得寧、鋅錳乃浦及賽普護汰寧可顯著降低此病原菌之孢子發芽，另雨季時則應加強藥劑防治，以降低田間病原菌之侵染，達到防治本病害之目的。以上本研究報導沙漠玫瑰葉斑病之病徵表現、病原形態、生長適溫及藥劑敏感性等資料，可作為將來病害鑑定及防治時之參考。

引用文獻

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Corynespora cassiicola Causes Leaf Spot on *Adenium obseum* in Taiwan

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Abstract

Huang, C. W., S. J. Zeng, J. H. Huang, and J. N. Tsai. 2025. *Corynespora cassiicola* causes leaf spot on *Adenium obseum* in Taiwan. J. Taiwan Agric. Res. 74(4):485–495.

In July 2022, leaf spot disease of desert rose (*Adenium obseum*) occurred in the Changhua area, with symptoms showing brown, round, or irregular-shaped lesions on leaves. In severe cases, the affected leaves yellowed and dropped off prematurely. In this study, a fungus was isolated from the diseased leaves and cultured on potato dextrose agar (PDA) medium. The colony was grayish-white to gray, with the reverse side of the medium turning brown to black. The conidia of this fungus were cylindrical or barrel-shaped, straight or slightly curved, light brown, with multiple septa, and blunt at the apex. Based on the symptoms of the disease, the morphological characteristics of the pathogen, and molecular sequencing identification results, this fungus was identified as *Corynespora cassiicola*. Artificial inoculation tests confirmed that *C. cassiicola* was pathogenic to desert rose leaves, and Koch's postulates were completed, proving this fungus to be the causal agent of desert rose leaf spot disease. The optimal temperature was 24−32℃ for mycelial growth of *C. cassiicola* and 20−28℃ for spore germination. Mycelial growth of the pathogen was effectively inhibited by Cyprodinil + Fludioxonil, Boscalid, and Oxine-copper. Spore germination of the pathogen was inhibited by Oxine-copper, Mancozeb, and Cyprodinil + Fludioxonil based on fungicide screening conducted *in vitro*. The findings of this study on the disease symptoms, pathogen morphology, growth temperature, and fungicide sensitivity can serve as a reference for future disease identification and control efforts. This is the first report of leaf spot disease on desert rose caused by *C. cassiicola* in Taiwan.

Key words: Desert rose (*Adenium obseum*), Leaf spot, *Corynespora cassiicola*, Fungicides screening.

Received: May 15, 2025; Accepted: August 21, 2025.
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台灣農業研究 (J. Taiwan Agric. Res.) 74(4):497–513 (2025)
https://doi.org/10.6156/JTAR.202512_74(4).0008

研究報告

紅龍果濕腐病菌 *Gilbertella persicaria* 之生物特性與發病條件之探討

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摘要

林筑蘋、徐敏記、蔡志濃、范浚維、黃晉興。2025。紅龍果濕腐病菌 *Gilbertella persicaria* 之生物特性與發病條件之探討。台灣農業研究 74(4):497–513。

由 *Gilbertella persicaria* 引起的紅龍果濕腐病是影響臺灣紅龍果產業的重要病害之一，然而其菌株之生物特性與致病性尚未完全釐清。自 2009–2024 年，本研究於全臺 11 個縣市、23 鄉鎮，共 90 處之田間與 1 處市場，採集罹病紅龍果樣本，分離得 212 株 *G. persicaria*，全部都為負交配型。此外，亦自田間與市場之罹病草莓與桃子，各分離得 1 株 *G. persicaria*，皆為正交配型，顯示 *G. persicaria* 在臺灣同時存在有 2 種交配型。為探討 *G. persicaria* 不同寄主來源菌株之差異，進一步進行形態學、病原性及分子親緣關係分析。結果顯示 (1) 形態方面，紅龍果分離株孢囊壁片數平均值顯著高出模式菌株 (type strain) 2.1–2.6 片；(2) 病原性測試顯示，僅紅龍果分離株才能在紅龍果果實上引起典型的濕腐病病徵，且病徵形態與病斑大小及 *G. persicaria* 寄主來源有關，而與交配型無關；(3) 利用內轉錄間隔區 (internal transcribed spacer; ITS)、actin 及 28S rDNA large subunit 等 3 個 DNA 片段的序列建構之親緣關係樹分析結果顯示，紅龍果分離株可獨自成為一支序群。由形態、病原性及親緣關係來看，紅龍果分離株具有獨特性。在發病條件試驗中，最適發病溫度為 28–36℃，最適發病相對濕度 (relative humidity; RH) 為 80% 以上，16℃以下或 40℃以上則不發病。進一步於田間將不同齡期果實接種 *G. persicaria*，並於成熟採收後貯放於 25–32℃環境中以觀察病害發展情形。結果發現以採收後接種之果實其發病率與罹病度最高，顯示該菌之主要感染時期為果實成熟階段，特別是在採收時期。

關鍵詞：孢囊壁分裂數、交配型、病原性、親緣關係、感染時機。

前言

由濕腐病菌 *Gibertella persicaria* (E.D. Eddy) Hesselt 引起的紅龍果濕腐病，不僅在臺灣 (Lin *et al*. 2014)，也在中國大陸、泰國、墨西哥、日本及越南等地，皆引起紅龍果的損失 (Taba *et al*. 2011; Guo *et al*. 2012; Carrillo-Fasio *et al*. 2022; Ruangwong *et al*. 2022; Vo *et al*. 2024)。紅龍果濕腐病之病徵包括田間紅龍果花器、幼果腐爛，甚至莖部腐爛 (Taba *et al*. 2011)；果實成熟採收後，於適合發病的環境中發病，罹病果實出現大面積水浸狀腐爛，並且擴散蔓延至鄰近果實。在夏季時，紅龍果濕腐病可於短期內造成大量花苞、幼果折損或果實腐爛；花或成熟果實一旦感染，約於接種 1 d 後即開始出現典型水浸狀腐爛病徵，2 d 後可看到黑色之 *G. persicaria* 孢囊柄與白色菌絲 (Lin *et al*. 2014, 2020)。

除感染紅龍果外，濕腐病菌 *G. persicaria*

投稿日期：2025 年 6 月 4 日；接受日期：2025 年 10 月 1 日。
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廣泛存在於田間與環境，如土、植株殘體甚至污水處理廠，亦可發現其蹤跡 (Hesseltine 1960; Ginting *et al*. 1996)。最早記錄的 *G. persicaria* 寄主為桃 (Hesseltine 1960)，後續發現其亦可感染梨、番茄、蘋果 (Mehrotra 1964a, 1964b; Chen *et al*. 2024)、木瓜 (Cruz-Lachica *et al*. 2016; de Almeida Souza *et al*. 2024)、草莓 (Zhang *et al*. 2020)、李子 (Pinho *et al*. 2014)、麵包樹、西印度櫻桃及腰果 (de Almeida Souza *et al*. 2024) 等，寄主範圍逐漸增加。其中，桃不但是最早被發現的寄主，也是最廣為所知的寄主 (Benny 1991; Ginting *et al*. 1996)。

Gilbertella persicaria 屬於真菌界 (Fungi)、接合菌門 (Zygomycota)、接合菌綱 (Zygomycetes)、毛黴目 (Mucorales)、Gibertellaceae (Benny 1991)。目前 *Gilbertella*屬僅有 *G. persicaria* 一個種 (Benny 1991)。*G. persicaria* 生殖方式包括有性與無性 2 種方式，其中行有性生殖時為異宗交配 (heterothallic)，交配型分為正交配型 (+) 與負交配型 (−) 2 種，當兩者同時存在並且接觸時才能產生接合子 (O'Donnell *et al*. 1977)。相較於 *Rhizopus* 屬與 *Choanephora* 屬，*Gilbertella* 屬最大的差異為孢囊 (sporangia) 具有孢囊壁 (sporangial wall)，且孢囊壁上具有 1 條縱向裂縫 (longitudinal suture)，成熟時孢囊壁由縱向裂縫分裂成 2 片，並且釋放孢囊孢子 (sporangiospores) (Hesseltine 1960; Benny 1991)。根據組織學研究，一旦 *G. persicaria* 侵染寄主後即可快速崩解寄主細胞，僅需 48 h 即可發展出孢囊產生下一代感染源，屬死體營養型 (necrotrophic lifestyle；或稱殺生型) (Cruz-Lachica *et al*. 2018)。關於 *G. persicaria* 的病原性研究方面，已知高溫有助於感染桃，且有助菌絲生長與產生孢囊 (Ginting *et al*. 1996)，其他環境影響因子如營養供應有無或者濕度高低等，皆會影響孢囊孢子發芽率 (Ginting *et al*. 1996; Taba *et al*. 2022)。

然而特別的是，觀察 *G. persicaria* 紅龍果分離株，發現其孢囊壁可分裂為 4 片以上 (Taba *et al*. 2011; Lin *et al*. 2014)；在比較桃與紅龍果分離株對紅龍果果實的致病力差異研究中，也發現兩分離株致病力有不同的趨勢 (Lin *et al*. 2014)；另一方面，內轉錄間隔區 (internal transcribed spacer; ITS) 序列分析也顯示紅龍果分離株與模式菌株 (type strain) 有些微差距 (Taba *et al*. 2011; Guo *et al*. 2012; Lin *et al*. 2014)。關於 *G. persicaria* 感染紅龍果的病原研究方面，觀察到 *G. persicaria* 可於罹病組織表面產生大量的孢囊，推測在田間主要以孢囊孢子進行感染，並且隨風雨散播孢囊孢子 (Ruangwong *et al*. 2022)。與在其他寄主上的研究相似的是，高溫有助於 *G. persicaria* 紅龍果分離株的菌絲生長 (Lin *et al*. 2014)；或是有學者提出建議，紅龍果儲放在低溫與未覆蓋的容器內，可降低 *G. persicaria* 引起的病害 (Guo *et al*. 2012)。目前對於 *G. persicaria* 感染紅龍果時的影響因子瞭解仍有限，仍有許多需要釐清之處。

本篇研究目的即在於瞭解 *G. persicaria* 紅龍果分離株之特殊性與病原性。本研究蒐集田間或市場 2009–2024 年之 *G. persicaria* 分離株，透過配對檢測試驗瞭解其在田間交配型存在狀況，也蒐集不同寄主分離株，以確認是否存在病原性與親緣關係差異；進一步透過人工接種紅龍果的方式，確認病害發展時對環境因子如溫度與濕度的需求，並且以田間幼果進行接種與調查成熟採收後發病狀況，確認採後濕腐病主要的感染時機。期望藉此更瞭解 *G. persicaria* 紅龍果分離株的生態特性，有助發展合適的防治方法，降低紅龍果濕腐病引起的損失。

材料與方法

配對型測試

自 2009–2024 年，於臺灣紅龍果產地，包括臺中市 (外埔、霧峰及后里，共 3 鄉鎮、17 處)、南投縣 (集集、中寮、名間、草屯及魚池，共 5 鄉鎮、23 處)、彰化縣 (二林與埔心，共 2 鄉鎮、27 處)、雲林縣 (水林，共 1 鄉鎮、1 處)、嘉義縣 (太保，共 1 鄉鎮、1 處)、臺南縣 (東山，共 1 鄉鎮、2 處)、高雄市 (岡山、鳳山、大寮及美濃，共 4 鄉鎮、5 處)、屏東縣 (長治、萬丹及內埔，共 3 鄉鎮、7 處)、宜蘭縣 (三星，共 1 鄉鎮、3 處)、臺東縣 (臺東市，共 1 鄉鎮、2 處) 及澎湖縣 (七美，共 1 鄉鎮、2 處) 等，共 11 個縣市、23 鄉鎮、90 處之田間，以及臺中市果菜市場 (1 處)，蒐集出現紅龍果濕腐病

病徵之花、幼果、莖或果實，並以組織分離方式分離病原菌，共計 212 株。詳細分離與保存方式如 Lin *et al*. (2014) 所示。本研究使用的菌株資料如表 1 所示，部分菌株寄存於食品工業發展研究所生物資訊保存中心。CBS190.32、CBS246.59 及 CBS247.59 等 3 菌株係經農業部動物植物防疫檢疫署同意輸入臺灣之菌株，特定物品、植物及其產品輸入許可證號碼為 “103-F-003-R”。交配型測定用菌株 CBS246.59、CBS247.59 及供試菌株先於馬鈴薯葡萄糖瓊脂 (potato dextrose agar (PDA); BD Difco^{TM}, Franklin Lakes, NJ, USA) 培養 2 d，以直徑約 0.5 cm 打孔器取菌落邊緣處。交配型測定用菌株正 (+) 或負 (−) 與供試菌株以相距約 2 cm 對峙的培養方式，菌絲塊朝下置於同一個 3.3% 麥芽瓊脂培養基 (malt-extract agar, MEA; BD Difco^{TM}, Franklin Lakes, NJ, USA) 下；單獨培養則直接放分離株於 MEA 上。培養於 24–28℃全光照環境下 2–3 d，以顯微鏡 (Olympus BX40, Olympus, Tokyo, Japan) 觀察接合子產生狀況。MEA 內容物為 1 L dH_2O 含有 12.75 g 麥芽萃取物 (malt extract; Difco, Kansas, USA)、2.75 g 糊精 (dextrin；聯合化學試藥，臺灣新竹市)、2.35 mL 丙三醇 (glycerol；島久藥品株式會社，日本大阪市)、0.78 g 蛋白腖 (peptone; Difco, Lawrence, KS, USA) 及 15 g 洋菜凍 (agar; Difco, Lawrence, KS, USA) 等。

孢囊壁形態觀察

沾取供試菌株的孢囊孢子，以輕碰觸邊緣的方式將供試菌株移植於約 0.5 × 0.5 × 0.3 cm^3 大小之 PDA 塊 4 個邊。PDA 塊放置於載玻片上，於移植後蓋上蓋玻片，移入盛有紙巾與無菌水的玻璃培養皿濕室 (moist chamber) 中，於室溫 26–28℃全黑暗培養 1–2 d 後，以鑷子逐一夾取供試菌株各個孢囊，於解剖顯微鏡 (Olympus SZX12, Olympus, Tokyo, Japan) 下觀察孢囊壁數量。每個菌株取 50 個孢囊。

病原性測試

於南投或臺中市場購置白肉紅龍果 (*Hylocereus undatus*)、草莓 (*Fragaria* × *ananassa* cv. ‘Toyonoka’)、桃 (*Prunus persica*, white peach) 及新興梨 (*Pyrus pyrifolia* cv. ‘Shinko’) 等果實。接種源製備與傷痍處理方式如 Lin *et al*. (2014) 所述，不同果實病原性測試中使用不同懸浮液濃度：紅龍果與梨試驗使用 1 × 10^6 spores mL^{-1}、草莓試驗使用 1 × 10^4 spores mL^{-1} 以及桃試驗使用 1 × 10^5 spores mL^{-1}，對照組以水取代孢子懸浮液。果實接種點表面以蟲針進行傷痍處理，以 5 mm 棉花球浸泡入各供試菌株孢囊孢子懸浮液後，放置於接種點表面，以膠帶固定，然後將果實移入盛有紙巾與無菌水消毒過的保鮮盒中 (35 × 21 × 10 cm^3)，使相對濕度 (relative humidity; RH) > 99%。上述含有接種果實的保鮮盒靜置於室溫 (25–32℃)、黑暗培養，3 d 後量測接種點病斑直徑，取長與寬之平均直徑作為每一個病斑之尺寸。每供試菌株於每果實之病原性測試至少有 6 個接種點，並且每個病原性測試重複至少 2 次。

統計分析

試驗結果資料，以統計分析軟體 SAS 7.1 進行變方分析 (analysis of variance; ANOVA)，並以最小顯著性差異 (least significant difference; LSD) 測驗在 $P < 0.05$ 顯著水準下，比較處理間平均值之差異。

建構親緣關係樹

基因序列增幅與定序

將萃取自供試菌株之 gDNA 以特定引子對進行聚合酶鏈鎖反應 (polymerase chain reaction; PCR)，增幅的序列包括 ITS (引子對 ITS5: GGAAGTAAAAGTCGTAACAAGG/ITS4: TCCTCCGCTTATTGATATGC; White *et al*. 1990)、actin (*ACT*) (引子對 Act 1: TGGGACGATATGGAIAAIATCTGGCA/Act 4R: TCITCGTATTCTTGCTTIGAIATCCACAT; Voigt & Wöstemeyer 2000) 及部分 28S rDNA large subunit (*LSU*) 基因之片段 (引子對 LROR: ACCCGCTGAACTTAAGC/LR6: CGCCAGTTCTGCTTACC; Vilgalys & Hester 1990; Moncalvo *et al*. 1995)。PCR 反應混合液 (20 μL) 包含 10 μL 耐熱聚合酶預混液 (*Taq* DNA polymerase 2× Master Mix RED, Ampliqon, Denmark)、1 μL

gDNA、0 μM 引子對各 1 μL 及 7 μL ddH_2O；所使用之引子對與反應條件如各文獻所列。以瓊脂膠體電泳確認 PCR 增幅產物為單一明顯條帶後，送交源資國際生物股份有限公司 (Tri-I Biotech Inc., New Taipei, Taiwan) 進行定序。

序列比較與親緣關係樹分析

序列相似度比較用的序列資料與親緣關係樹用的參考菌株 (reference strain) 序列資料，皆由美國國家生物技術資訊中心 (National Center for Biotechnology Information; NCBI) 資料庫取得。建構親緣關係樹時，將各基因之序列分別透過 MEGA X 軟體 (Kumar *et al*. 2018) 以 Clustal W (Thompson *et al*. 1994) 進行初步排列分析 (alignment)，再透過人工方式進行序列的微調與確認最終各基因片段用以構築單一 (ITS) 或 3 基因 (ITS + *ACT* + *LSU*) 之多基因親緣關係樹。親緣關係樹利用最大似然法 (maximum likelihood; ML) 與貝葉斯法 (Bayesian inference analysis; BI) 2 種演算方法進行構築，最適取代模型透過線上分析軟體 Smart Model Selection (Lefort *et al*. 2017) 與 MEGA X 軟體進行預測，其中最大似然法不分基因區段預測，貝葉斯法則依各基因區段單獨預測。最大似然法親緣關係樹之構築透過 MEGA X 軟體執行，並以啟發式 (heuristic method) 方法中 nearest neighbour interchange (NNI) 之方式搜索樹型，最終演化樹分支的支持度以拔靴法 (bootstrap) 進行 1,000 次取樣來檢驗。貝葉斯法之親緣關係樹利用 MrBayers v. 3.2.7 (Ronquist *et al*. 2012) 構築，建構過程採用馬可夫鏈蒙地卡羅 (Markov chain Monte Carlo; MCMC) 演算法，運行 1,000,000 代，每 1,000 棵樹取樣 1 次，前 25% 之樹屏除，剩餘之樹用以取得共同樹 (或稱一致樹；consensus tree)，並顯示後驗機率 (posterior probabilities; PP)，表示對此親緣樹分支的支持度，最終親緣關係樹以 FigTree v1.4.4 進行繪製。

溫度與濕度對感染的影響性試驗

以臺中外埔或彰化二林果園購入之白肉紅龍果果實為供試材料，紅龍果分離株 F210187 為供試菌株，製備 1 × 10^6 spores mL^{-1} 孢子懸浮液，以噴灑孢子懸浮液方式接種果實，直到每粒供試果實有水滴滑落，等待下一步測試。在溫度測試中，果實放置於如病原性測試所述的 RH > 99% 保濕保鮮盒內，放置於生長箱中，培養溫度分成 4、8、12、16、20、24、28、32、36 及 40℃ 等 10 處理。黑暗培養，4 d 後記錄罹病狀況。每個溫度處理 3 個果實，共重複 3 次。在濕度測試中，先在室內用風扇將果實表面風乾後，將果實放置於墊有止滑架塑膠籃中，放置於本所設計之可程式化濕度調節箱，RH 分成 50、60、70、80、90 及 100% 等 6 種處理，並以溫濕度偵測器 (HOBO, Onset Computer Corporation, Bourne, MA, USA) 置於箱內進行即時偵測。黑暗培養，溫度維持在 28℃，4 d 後記錄罹病狀況。每種濕度處理 5 個果實，共重複 3 次。

接種田間不同齡期的幼果

以農業部農業試驗所試驗田種植之白肉紅龍果為試驗植株，在花謝後第 3 天果實初現時即以牛皮紙袋套袋緊密包裹開口處進行套袋隔離，並將此時果實視為第 0 週大果實 (W0)。處理組分別於第 0 週 (W0)、第 1 週 (W1)、第 2 週 (W2)、第 3 週 (W3) 及第 4 週 (W4) 進行接種。果實接種後持續於植株上發育直至第 5 週成熟後採收，並於室溫 (25–32℃)、RH > 99% 條件下觀察病害發展。田間接種方式為移除紙袋後，噴灑 *G. persicaria* 菌株 F210187 孢子懸浮液 (1 × 10^6 spores mL^{-1}) 至果實表面水滴滴落為止，再以塑膠袋與牛皮紙袋覆蓋保濕遮光，4 d 後移除塑膠袋但保留牛皮紙袋持續隔離；採後組 (postharvest) 的接種方式相似，但果實為成熟後採收當日接種。罹病度統計方式如下述。每處理 15 粒果，試驗重複 4 次。正對照組為每次接種幼果時，都會同時接種 3 粒成熟果實，放置於同樣的田間環境，確定該次接種條件能成功引起病害，確認每次接種源的病原性無異常。負對照組為每次皆以無菌水取代孢子懸浮液，同時施布於 4 粒同株齡果實，同樣於果實成熟採收後觀察發病狀況。

罹病度統計

罹病級數 (disease index) 分為 5 級 (0：無病徵；1：罹病面積 < 25%；2：罹病面積 > 25%、< 50%；3：罹病面積 > 50%、< 75%；4：罹病面積 > 75%、< 100%)。罹病度 (disease severity) = $[(0n_0+1n_1+2n_2+3n_3+4n_4)/4N] \times 100\%$。$n_0$、$n_1$、$n_2$、$n_3$、$n_4$ 分別為 0–4 等各罹病級數的株數，N 為全部接種株數。發病率 (disease incidence) = (發病果總數/總接種果數) × 100%。利用統計分析軟體 SAS (EG7.1, Statistical Analysis System, USA) 進行 ANOVA，再以 LSD 測驗在 $P < 0.05$ 顯著水準下，比較處理間平均值之差異。

結果

Gilbertella persicaria 菌株配對型檢測

為瞭解 *G. persicaria* 菌株在臺灣紅龍果園區的交配型存在情況，蒐集 2009–2024 年臺灣各地田間罹染濕腐病之紅龍果果實或者花的樣本，以及極少部分市場果實，以組織分離方式純化菌株，共得 212 菌株。另外自苗栗田間草莓與臺中市場之進口水蜜桃亦分得 *G. persicaria* 菌株各 1 菌株，共 2 菌株。以上菌株，藉由與交配型測定用菌株 CBS246.59 (+) 與 CBS247.59 (−) 配對，測定各菌株之交配型。所有的紅龍果分離株在 MEA 獨自培養，或者與負交配型菌株 CBS247.59 (−) 對峙培養時，在培養基內無法觀察到產生接合子，而與正交配型菌株 CBS246.59 (+) 對峙培養時，則可以觀察得接合子，顯示此 212 株紅龍果分離株之配對型皆為負 (−)；另一方面，蒐集之 1 株草莓分離株與 1 株水蜜桃分離株則僅在與負交配型菌株 CBS247.59 (−) 對峙培養時才會觀察到產生接合子，顯示供試菌株皆為正交配型 (+)。

不同 *G. persicaria* 寄主分離株與交配型的病原性差異

為釐清不同分離來源與交配型對於造成紅龍果濕腐病的病原性是否具影響性，以 2 株分離自紅龍果 (負交配型)、1 株分離自草莓 (正交配型)、2 株分離自水蜜桃 (負與正交配型) 及 1 株分離自環境 (負交配型) 等，共 6 株不同分離來源與交配型的分離株作為供試菌株，接種紅龍果果實進行病原性測試。結果顯示，紅龍果菌株較其他分離株可造成顯著的病斑尺寸 (圖 1A)，且病徵呈深紅色、水浸狀斑 (圖 2A)，與典型的果實濕腐病病徵相同 (圖 2B)；其他分離株引起的病斑不僅局限，且水浸狀較輕微、病徵顏色較褐色，與典型的濕腐病病徵不同。交配型方面，與紅龍果分離株一樣為負交配型的桃分離株 CBS 190.32 其病徵狀況與紅龍果分離株不同，而與其餘來源分離株相同，顯示交配型對紅龍果果實的病原性 (pathogenicity) 與致病力 (virulence) 無關。

進一步，為瞭解在其他果實上是否也會出現分離株來源差異的趨勢，並且排除桃、草莓及環境等分離株病原性或致病力弱化的可能性，以草莓、桃等各分離株的原始寄主，及另一寄主之一的水梨等，共 3 種果實作為供試材料，再次接種以同樣的供試菌株進行病原性測試。在草莓上 (圖 1B)，病斑尺寸隨菌株而異，沒有出現因菌株寄主來源或交配型不同而異的趨勢。在桃上 (圖 1C)，2 株紅龍果分離株造成的病斑在統計上較其他分離株小，對桃的致病力較弱；草莓、桃及環境等 4 株分離株造成的病斑較大，對桃的致病力強，同時也排除分離株病原性弱化的可能性；病斑尺寸亦沒有出現受到菌株交配型影響的趨勢，而是與菌株來源相關。在梨上 (圖 1D)，各不同來源分離株間的病原性與致病力相似，病斑大小沒有出現受到菌株寄主來源或交配型而異的趨勢。

G. persicaria 各分離株的孢囊壁形態差異

為瞭解各分離株形態上的差異，觀察不同寄主來源分離株的孢囊壁形態 (表 1)。2 株紅龍果分離株的孢囊壁分裂數為平均數量為 4.7–5.0 片，最少 3 片，最多可達 8 片；1 株草莓分離株的孢囊壁為平均 3.2 片，最少 2 片，最多 5 片；1 株桃分離株的孢囊壁平均為 3.9 片，最少 2 片，最多 6 片；模式菌株的 1 株桃分離株，與分離自環境的 2 菌株，此 3 菌株的孢囊壁平均為 2.4–2.6 片，最少 2 片，最多 5

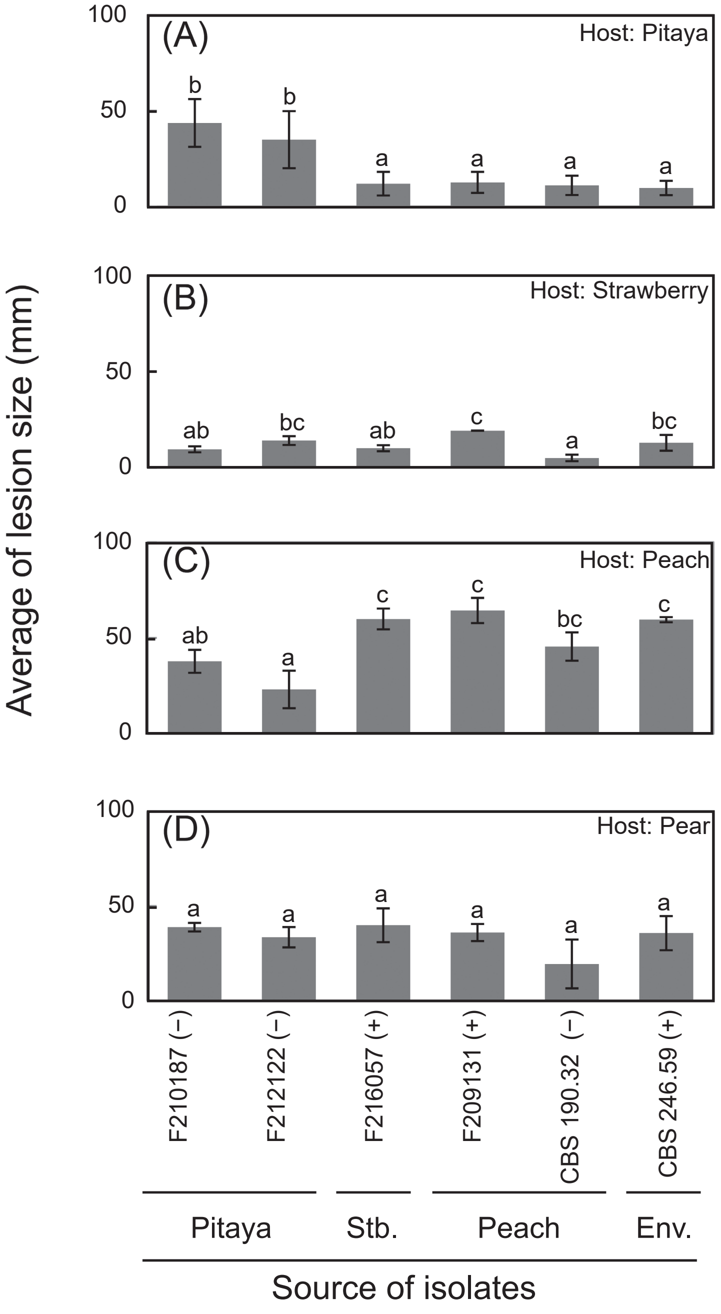

圖 1. *Gilbertella persicaria* 不同寄主來源菌株在不同果實上的病原性比較。(A) 白肉紅龍果、(B) 草莓、(C) 桃及 (D) 梨。菌株交配型以及來源標註在圖下方。“Stb.” 與 “Env.” 各自代表『草莓』與『環境』。不同字母代表顯著水準 $P < 0.05$ (雙因子變方分析，Fisher 的最小顯著差異性測驗)。誤差線代表 *SE*。

Fig. 1. Comparison of pathogenicity among *Gilbertella persicaria* isolates from different hosts on various fruits. (A) white-flesh pitaya, (B) strawberry, (C) peach, and (D) pear. Sources and mating types of isolates were indicated below the corresponding column. “Stb.” and “Env.” referred to “strawberry” and “environment”, respectively. Different letters indicated significant differences within each group at $P < 0.05$ (two-way analysis of variance; two-way ANOVA, Fisher’s least significant difference; LSD test). Error bars represent *SE*.

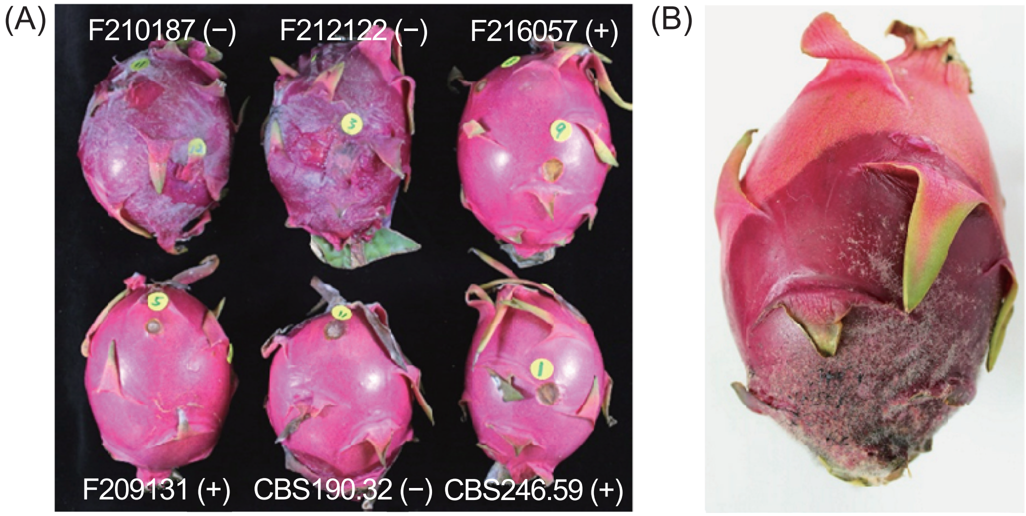

圖 2. (A) 人工接種與 (B) 自然感染發病之白肉紅龍果上 *Gilbertella persicaria* 引起的病徵。菌株 F210187 與 F212122 分離自紅龍果，F216047 來自草莓，F209131 與 CBS190.32 分離自桃子；CBS246.59 分離自環境。

Fig. 2. Symptoms of white-flesh pitaya caused by *Gilbertella persicaria* under (A) artificial inoculation and (B) natural infection. Isolate F210187 (−) and F212122 (−) originated from pitaya, F216057 (+) from strawberry, F209131 (+) and CBS190.32 (−) from peach, while CBS246.59 (+) was derived from an environmental sample.

表 1. 本研究 *Gilbertella persicaria* 菌株來源清單與孢囊壁數量。

Table 1. List of *Gilbertella persicaria* isolates source of this study and their numbers of sporangial walls.

Isolate	Substrate	Source	Mating type	Sporangial wall number			Note
				Avg. ± *SE*[z]	Min.	Max.	
F210187	Pitaya	Jiji, Nantou County	−	4.7 ± 0.1 d	3	6	
F212122	Pitaya	Zhongliao, Nantou County	−	5.0 ± 0.1 d	3	8	
F216057	Strawberry	Shitan, Miaoli County	+	3.2 ± 0.1 b	2	5	
F209131	Imported peach	Market in Taichung	+	3.9 ± 0.1 c	2	6	
CBS190.32	Peach	New York, USA (Benny 1991)	−	2.6 ± 0.1 a	2	4	Holotype
CBS246.59	Trickling filter plant	Ohio, USA (Hesseltine 1960)	+	2.4 ± 0.1 a	2	5	= NRRL 2352, mating standard isolate
CBS247.59	Soil	Taxas, USA (Hesseltine 1960)	−	2.5 ± 0.1 a	2	4	= NRRL 1546, mating standard isolate

[z] $N \geq 50$; different letters indicate significant differences within each group at $P < 0.05$ (analysis of variance (ANOVA), least significant difference (LSD) test).

片。紅龍果分離株的孢囊壁數量顯著高於模式菌株與其他分離株。

G. persicaria 各分離株的親緣關係

為瞭解各寄主分離株在親緣關係上的關係，以本研究中的 3 株紅龍果分離株為代表，1 株桃分離株，及 1 株草莓分離株等，共 5 株 (表 2)，與已知之 *G. persicaria* 菌株，包括分自於桃的模式菌株、前述配對型測定用菌株，及其他草莓分離株等，還有其他 6 菌種等，共 12 株作為參考菌株 (表 3)，並以 ITS、*ACT* 及 *LSU* 等 3 個片段的聯合序列建構最大概率親緣關係樹 (圖 3)。模式菌株與本研究桃分離株、環境分離株 (交配型測定用菌株)，及本

表 2. 本研究使用之 *Gilbertella persicaria* 分離株基本資料與序列號碼。

Table 2. Information and accession numbers of the *Gilbertella persicaria* isolates of this study.

Isolate	Location	Host	BCRC ID[z]	GenBank accession No.[y]		
				ITS	*ACT*	*LSU*
F210187	Market in Taichung	Pitaya	BCRC FU31440	MK301173	OP311904	OP243277
F212122	Zhongliao, Nantou County	Pitaya	BCRC FU31441	MK301174	OP311903	OP243278
F218061	Gangshan, Kaohsiung City	Pitaya	-	ON875323	OP311905	OP243279
F209131 (= F209130)	Market in Taichung	Peach	BCRC FU31439	MK301172	OP311901	OP243276
F216057	Shitan, Miaoli County	Strawberry	BCRC FU31442	MK301176	OP311902	OP243275

[z] IDs of the isolates deposited at Bioresource Collection and Research Center (BCRC) of Food Industry Research and Development Institute.
[y] Sequences of internal transcribed spacer (ITS), actin (*ACT*), and 28S rDNA large subunit (*LSU*) were included.

研究與其他研究之草莓分離株可成為一個 *G. persicaria* 支序群 (clade)，拔靴法支撐數值 (bootstrap support values) 為 90%，貝葉斯事後概率數值 (Bayesian posterior probabilities) 為 1；紅龍果分離株獨自形成另一個支序群，拔靴法支撐數值為 100%，貝葉斯事後概率數值為 1。親緣關係分析結果顯示，紅龍果分離株與 *G. persicaria* 親緣關係相近，但與包含模式菌株的其他 *G. persicaria* 菌株在親緣關係上仍具差異，形成穩定的 *G. persicaria* 支序群。

本研究紅龍果代表菌株 F210187、F212122 及 F218061 等共 3 菌株，其 ITS 與 *LSU* 基因之序列相似度皆為 100.0%；*ACT* 序列方面，F210187 與 F212122 之序列相似度為 100.0%，而 F210187 與 F218061 序列相似度亦達 99.9%。為排除此序列差異是地區的緣故，進一步比較來自不同地區的紅龍果分離株之序列差異。以 ITS 序列而言，本研究紅龍果分離株與中國大陸紅龍果分離株 (accession no. JQ951601) 相似度為 100%，與泰國紅龍果分離株 (LC654417) 相似度為 99.5%，與印度紅龍果分離株 (accession no. OR984746) 相似度則為 99.0%，而本研究紅龍果分離株與模式菌株 (桃分離株；accession no. ON875317) 之相似度為 98.3%；以本研究 3 株紅龍果分離株、4 株其他區域之紅龍果分離株，及其他 15 株不同來源菌株建築之親緣關係圖亦可發現，不同區域間的紅龍果分離株可獨自成為一支序群，拔靴法支撐數值為 95%，貝葉斯事後概率數值為 0.98 (圖 4)。以 *LSU* 序列而言，本研究紅龍果分離株與泰國紅龍果分離株 (accession no. LC655936) 之相似度為 100%，而兩者與模式菌株 (accession no. MH866729) 之相似度則為 99.0%。關於 *ACT* 序列之差異性比較，目前 NCBI 資料庫上尚未有其他紅龍果分離株之 *ACT* 供比較，本研究之紅龍果分離株與模式菌株之相似度則為 97.3%。由 ITS 與 *LSU* 的序列相似度可知，紅龍果分離株間的相似度高，且與模式菌株具差異性。

綜合病原性 (圖 1 與圖 2)、形態 (表 1) 及親緣關係 (圖 3 與圖 4)，*G. persicaria* 紅龍果分離株與 *G. persicaria* 模式菌株具有顯著的差異。

G. persicaria 紅龍果分離株感染紅龍果的重要因子

為瞭解溫度對 *G. persicaria* 重要感染體—孢囊孢子—的影響，以紅龍果分離株 F212122 與 F210187 共 2 菌株為代表供試，滴取孢囊孢子於 PDA 後，在不同溫度下培養，於不同時間點觀察孢囊孢子的發芽率。如表 4 所示，兩菌株孢囊孢子的發芽率相似。在 12℃以上，發芽率逐漸上升；28–36℃發芽率高，並在 7 h 內即高於 94.5 ± 0.9% 以上。其中，32℃的發芽率最高，而後逐漸下降，尤其 40℃以上發芽率驟降。

為瞭解溫度與濕度對 *G. persicaria* 感染紅龍果的影響，以紅龍果分離株 F210187 為代表供試，接種於成熟紅龍果果實，放置於不同溫度與濕度下，4 d 後觀察罹病度 (disease severity) 與發病率 (disease rate) 狀況 (圖 5)。在溫度方面 (圖 5A)，20℃以上罹病度與發病率逐漸上

表 3. 本研究參考菌株清單與序列代號。

Table 3. List and accession numbers of the reference species of this study.

Species	Isolate	GenBank accession No.[z]			Note
		ITS	*ACT*	*LSU*	
Gilbertella persicaria	CBS190.32 (T)	ON875317	OP311898	MH866729	Zhang *et al*. (2020) and this study
	CBS246.59	JN206222	OP311899	OP243274	Walther *et al*. (2013) and this study
	dg-seq	JQ951601	-	-	Guo *et al*. (2012)
	GP1	OR984746	-	-	-
	HLGXF	ON248470	-	-	-
	HP183	KT213049	-	-	Cruz-Lachica *et al*. (2016)
	PSU-FR04	LC654417	-	-	Ruangwong *et al*. (2022)
	CBS247.59	ON875318	OP311900	MH869391	Zhang *et al*. (2020) and this study
	SB2018-203	MT019675	MN970197	MT010345	Zhang *et al*. (2020)
	SB2019-09	MT019676	MN970198	MT010346	Zhang *et al*. (2020)
	SB2019-10	MT019677	MN970199	MT010347	Zhang *et al*. (2020)
Blakeslea trispora	CBS130.59	JN206227	AJ287143	MH869368	Zhang *et al*. (2020)
Poitrasia circinans	CBS153.58 (type strain)	NR_145288	AJ287188	NG_057934	Zhang *et al*. (2020)
Choanephora infundibulifera	F214060	ON875324	OP311906	OP243280	This study
Cokeromyces recurvatus	CBS158.50 (type strain)	NR_077172	AJ287150	AF113454	Lee *et al*. (2018)
Mucor flavus	CBS234.35 (type strain)	JN206051	EF203689	NG_057921	Hyde *et al*. (2014)
Rhizopus schipperae	ATCC 96514 (type strain)	DQ641323	AB512229	NG_059417	-

[z] ITS: internal transcribed spacer; *ACT*: actin; *LSU*: 28S rDNA large subunit.

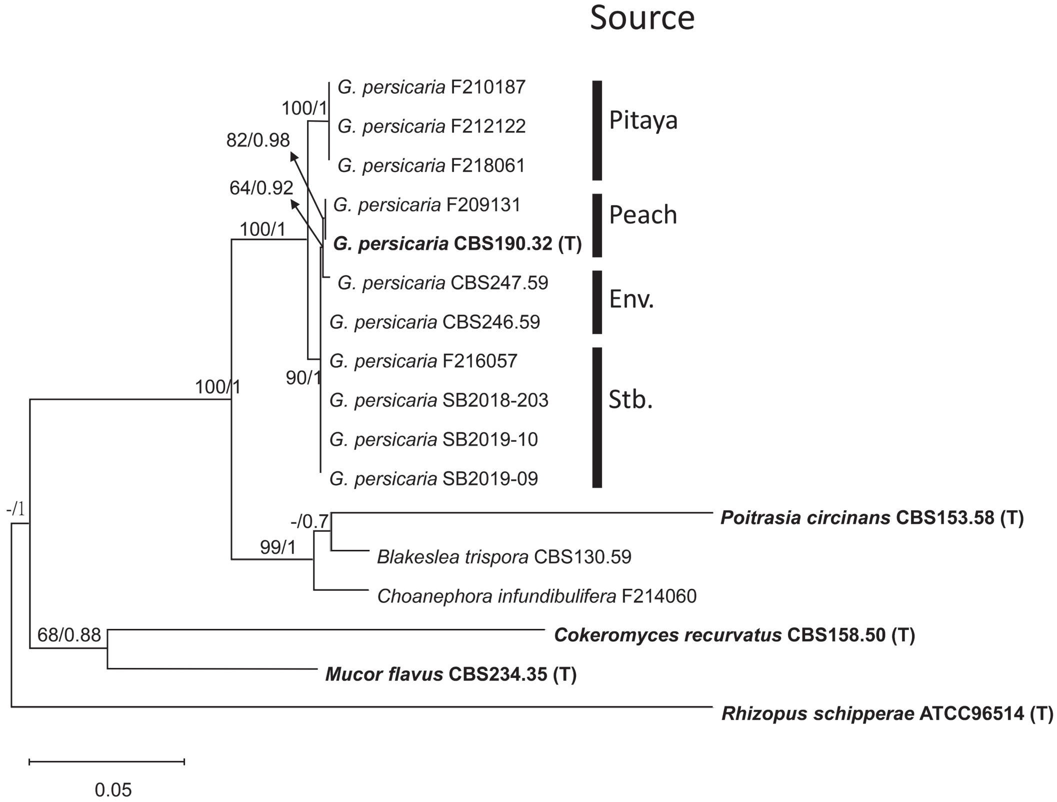

圖 3. *Gilbertella persicaria* 與其相近種以 internal transcribed spacer (ITS)、actin (*ACT*) 及 28S rDNA large subunit (*LSU*) 共 3 序列構築之最大概率親緣關係圖。比例尺代表每個核苷酸位置的 0.05 預期置換數。模式菌株以粗體字標示。菌株來源標註在圖右方。“Env.” 與 “Stb.” 各自代表『環境』與『草莓』。

Fig. 3. Maximum likelihood phylogenetic tree of *Gilbertella persicaria* and related texa based on the combined sequences of internal transcribed spacer (ITS), actin (*ACT*), and 28S rDNA large subunit (*LSU*). The bar indicates 0.05 expected changes per site. Type strains (T) are displayed in bold. Isolation source are labeled on the right: Env.: environment.; Stb.: strawberry.

升，28–36℃發病嚴重，其中以 28℃發病率最高，可達 100 ± 0.0%，罹病度為 36.1 ± 5.6%，而 32℃發病率次之，為 77.8 ± 22.2%，但罹病度最高，達 69.5 ± 14.7%；40℃驟降，發病率與罹病度皆降為 0%。在濕度方面 (圖 5B)，RH 50–70% 發病狀況相似，發病率在 75.0 ± 9.6% 至 80.0 ± 8.2% 間，罹病度在 25.0 ± 8.1% 至 31.0 ± 6.6% 間；RH 80% 以上發病率提升至 90.0 ± 5.8%，罹病度則為 35.0 ± 7.7%；RH 90% 以上發病率緩慢的提升為 95.0 ± 5.0%，而罹病度顯著的提升為 62.0 ± 13.1%；RH 100% 發病率與罹病度最高，個別為 100 ± 0.0% 與 78.0 ± 9.9%。

田間 *G. persicaria* 感染時機對採後罹病度影響

為瞭解紅龍果採後濕腐病的感染時機為何，以紅龍果分離株 F210187 為代表供試，於田間接種在 0–4 wk 與已成熟採收等不同果齡的白肉紅龍果，直到各組果實都成熟採收後，將果實放置於 28–32℃，RH > 95% 的環境，4 d 後觀察罹病度與發病率狀況 (圖 6)。田間幼果接種後，皆沒有出現濕腐病徵，待成熟採收並貯藏在適合的環境下才發病。果實在 0–3 wk 果齡受感染時，發病率在 1.7–8.6% 間，罹病度則在 0.8–4.2% 間；4 wk 果齡受感染者，

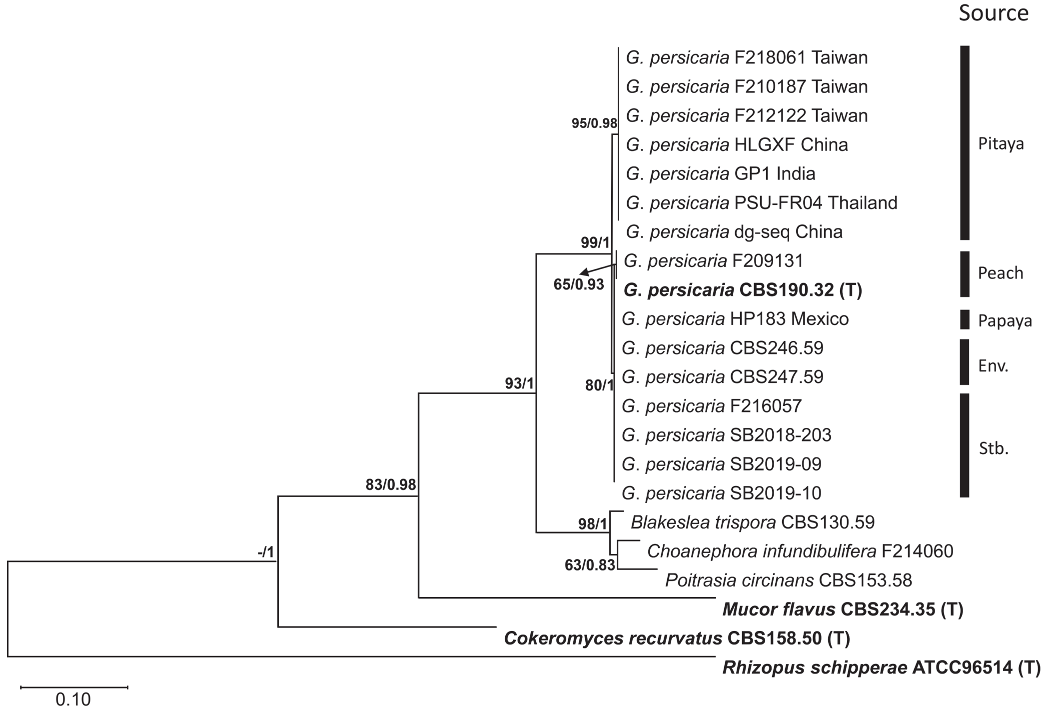

圖 4. *Gilbertella persicaria* 與其相近種以 ITS 序列構築之最大概率親緣關係圖。比例尺代表每個核苷酸位置的 0.05 預期置換數。模式菌株以粗體字標示。紅龍果菌株之原地理位置標註在分離株代碼後。菌株來源標註在圖右方。“Env.” 與 “Stb.” 各自代表『環境』與『草莓』。

Fig. 4. Maximum likelihood phylogenetic tree of *Gilbertella persicaria* related taxa constructed based on the ITS region. The scale bar indicates 0.05 expected changes per site. Type strains (T) are displayed in bold. The geographic origins of the pitaya isolates were annotated following its isolate ID. Isolation source are labeled on the right: Env.: environment; Stb.: strawberry.

採後病害狀況微幅提高，發病率為 13.8 ± 4.7%，罹病度為 6.0 ± 2.4%；成熟採收果受感染者，病害狀況大幅提高，發病率為 91.2 ± 5.2%，罹病度為 56.3 ± 7.1%，顯示紅龍果採後濕腐病的感染時間點在成熟後期。每次用以做正對照的果實皆出現濕腐病病徵，確認每次接種皆為有效接種；以水取代孢子懸浮液的負對照組果實，於採收後皆未出現濕腐病病徵，排除自然界之 *G. persicaria* 病原感染干擾。

討論

本研究蒐集 2009–2024 年樣本，經過測試確定 *G. persicaria* 在臺灣存在正 (+) 與負 (−) 交配型菌株，惟感染紅龍果的 *G. persicaria* 菌株目前發現 100% 皆為負交配型，尚未發現正交配型存在 (表 2)。從病原性測試結果可知，與紅龍果分離株同樣為負交配型的菌株 CBS 190.32 亦不會引起較明顯的果實病徵 (圖 1)，顯示病原性與交配型無關，排除紅龍果田間 *G. persicaria* 負交配型優勢的是因為病原性的關係。另外，造成日本紅龍果莖腐與果腐病病徵的 *G. persicaria* 為正交配型 (Taba *et al*. 2011)；在桃上，正負交配型不影響病原性 (Ginting *et al*. 1996)，以上皆支持正交配型不影響感染紅龍果病原性的推理。為什麼臺灣田間紅龍果濕腐病病原僅有 *G. persicaria* 負交配型的原因仍未知。行有性生殖進行遺傳物質交換產生接合子，有利族群遺傳物質多變異性。實驗室內對峙培養測試，紅龍果分離株 (−) 與草莓分離

表 4. 溫度對 *Gilbertella persicaria* 菌株 F210187 與 F212122 孢囊孢子發芽率的影響。

Table 4. Effect of temperature on sporangiospore germination of *Gilbertella persicaria* isolates F210187 and F212122.

	Sporangiospore germination rate (%)[z]					
	F210187			F212122		
Isolate (℃)	7 h	24 h	48 h	7 h	24 h	48 h
4	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
8	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
12	0 ± 0	0 ± 0	43.7 ± 6.7	0 ± 0	100 ± 0	UC
16	0 ± 0	94.0 ± 1.2	UC	0 ± 0	UC[y]	UC
20	0 ± 0	100 ± 0	UC	0 ± 0	UC	UC
24	24.3 ± 6.6	UC	UC	46.5 ± 7.9	UC	UC
28	94.5 ± 0.9	UC	UC	100 ± 0	UC	UC
32	100 ± 0	UC	UC	100 ± 0	UC	UC
36	88 ± 3.1	UC	UC	100 ± 0	UC	UC
40	0 ± 0	0.8 ± 0.8	1.8 ± 1.8	29.5 ± 4.2	UC	UC

[z] Spore suspensions of each *G. persicaria* isolate were dropped onto potato dextrose agar (PDA) plates and incubated in the dark at various temperatures. After 7 h, germination rates were calculated by counting 200 spores per replicate. A spore was considered germinated if its germ tube exceeded the spore radius. Each isolate was tested in triplicate.

[y] UC indicates "uncountable" due to excessive hyphal growth.

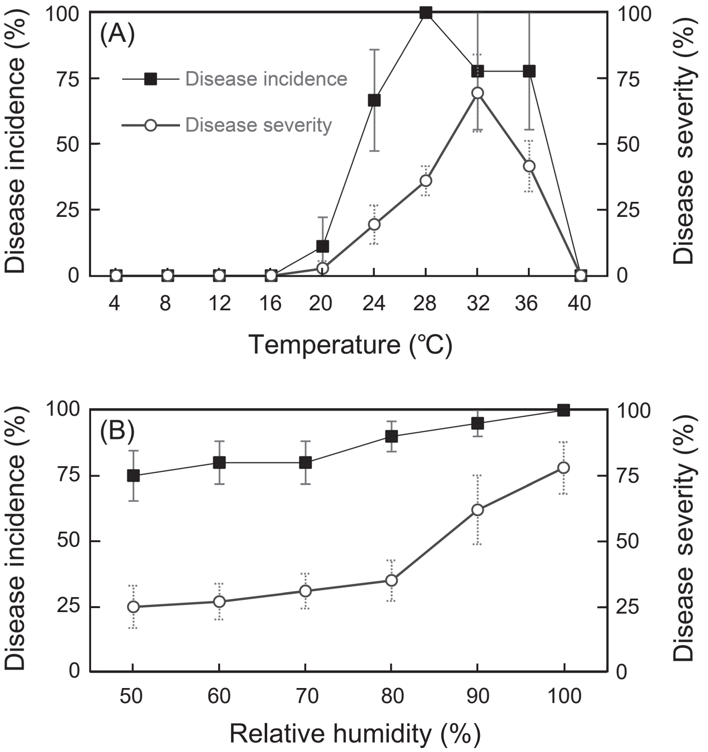

圖 5. (A) 溫度與 (B) 相對濕度對 *Gilbertella persicaria* 引起之紅龍果果實濕腐病病害發展影響。誤差線代表 *SE*。

Fig. 5. Effect of (A) temperature and (B) related humidity (RH) on disease development of pitaya fruit wet rot caused by *Gilbertella persicaria*. Error bars represent *SE*.

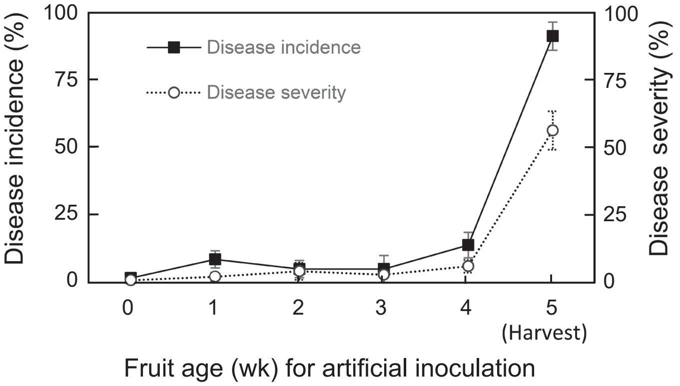

圖 6. 紅龍果果齡在田間感染 *Gilbertella persicaria* 對於果實採收後濕腐病發展的影響。誤差線代表 *SE*。

Fig. 6. Effect of field artificial infection timing by *Gilbertella persicaria* on the development of postharvest wet rot in pitaya fruits. Error bars represent *SE*.

株 (+) 可行有性生殖產生接合子 (未顯示之資料)，但 *G. persicaria* 在臺灣田間是否可自然配對產生有性世代仍有待證實。

Gilbertella persicaria 的寄主範圍廣泛，本研究特別檢測各個來源寄主病原株是否有病原性差異。在感染紅龍果的測試中 (圖 1)，唯紅龍果分離株可引起顯著典型的紅龍果濕腐病病徵相同 (圖 2) (Taba *et al*. 2011; Guo *et al*. 2012; Lin *et al*. 2014)，而桃、草莓及其他分離株引起的病斑則否，顯示唯紅龍果分離株對紅龍果具有獨特的病原性。進一步以 *G. persicaria* 模式菌株原始寄主一桃 (Hesseltine 1960) 做病原性測試 (圖 1C)，顯示相較於而桃、草莓及其他分離株，紅龍果分離株對桃的病原性有較低的趨勢。

在臺灣與日本的 *G. persicaria* 紅龍果分離株孢囊壁紀錄為4片以上 (Taba *et al*. 2011; Lin *et al*. 2014)。然而，根據文獻，*G. persicaria* 模式菌株的孢囊壁為 2 片 (Benny 1991)。統計各分離株間孢囊壁數量差異 (表 1)，可知以紅龍果分離株的平均數量最高，供試的 2 菌株平均為 4.7–5.0 片，而模式菌株與其他環境分離株等則為 2.4–2.6 片，兩者在孢囊分裂數上相距高達 2.1–2.6 片，在統計上具顯著差異。然而，本研究中分離自進口桃與草莓分離菌株的孢囊壁數量與模式菌株等亦差距達 0.8–1.4 片，並且在統計上具有顯著性 (表 1)，但病原性 (圖 1 與圖 2) 與分子親緣關係樹 (圖 3) 中仍與模式菌株屬相同類群。雖然紅龍果分離株與模式菌株的孢囊壁分裂數差距大，但孢囊壁的數量多寡要成為區分不同種的特徵仍有疑慮。

紅龍果分離株在親緣關係上與其他分離株不同，且具有高拔靴法支撐數值支持此論述 (圖 3)，且此紅龍果分離株序列上與模式菌株具差異之現象非僅限於臺灣的菌株 (圖 4)。這種不同寄主來源分離株序列具差異的現象在巴西的 *G. persicaria* 分離株中也出現類似的趨勢 (de Almeida Souza *et al*. 2024)。綜上結果顯示，跨區域紅龍果分離株彼此間具高度相似性，並相對區別於自桃分離之模式菌株，推測可能具有共同的分子特徵或獨特性。

關於紅龍果分離株的病原性，本研究進一步確認，*G. persicaria* 紅龍果分離株感染採後紅龍果時受到至少溫度與濕度及影響 (圖 5)，最適合發病的溫度為 28–36℃，濕度為 RH 80% 以上，在 16℃以下的低溫與 40℃以上的高溫不發病，RH 70% 以下發病率與罹病度會顯著降低。其中若接種後放置於低溫下 (16℃)，即便給予高濕度 (RH > 99%) 者也不會發病；若放置於合適溫度下 (28–30℃)，給予低濕度 (RH < 70%) 則病害度僅降低而非不發病。顯示溫度對於病害發展的影響性大於濕度。由以上可知，為避免紅龍果濕腐病，低溫 (16℃) 貯藏為較合適的貯藏條件；若無法維持低溫，則低濕度 (RH < 70%) 為次之的貯藏選擇。果實持續放在 40℃以上的高溫不合一般

貯藏習慣，除了考慮果實品質可能因為高溫劣質化外，一旦從高溫回到室溫時濕腐病害可能會再恢復 (Ginting *et al*. 1996)，而且高溫處理過的樣本會提高罹染 *G. persicaria* 的發病率 (Taba *et al*. 2022)。

從田間幼果期感染時機試驗可知 (圖 6)，幼果期接種 *G. persicaria* 的果實於採收後濕腐病發病率低，而越接近成熟時接種，則後續的濕腐病發病率高。成熟果較幼果感病的原因仍有待釐清。另外，由本試驗可知，田間幼果接觸到 *G. persicaria* 時仍可能會造成後續貯藏期果實發病，但發病比例低，最主要的感染期為果實成熟後期，尤其是採收後。此狀況符合實際觀察，田間果實接近成熟期時，若遇到連續下雨則後續容易發生嚴重的濕腐病。可能原因是此時的果實感病性高，若雨中含有隨雨傳播的孢囊孢子，則發病機率高；或是果實成熟後，於採收過程中碰到 *G. persicaria* 污染的雨水、器具及包裝紙袋等，導致貯藏後發病嚴重。另外，*G. persicaria* 的感染源包括田間與包裝場的土壤及地面的殘體，或是包裝場用以冷卻果實的用水中 (Ginting *et al*. 1996)。在臺灣，紅龍果採收後常以水再處理過以擦拭掉煤煙，該擦拭用的水若被 *G. persicaria* 污染，可能成為採收後的重要感染源頭。

幼果期接種 *G. persicaria* 不會有明顯病徵，直到果實成熟採收後放置於高溫高濕環境下才會出現典型紅龍果濕腐病病徵。病原菌潛伏感染時，菌株常以附著器 (appressoria) 附著於植物表面，等待時機成熟後再進一步侵入發展菌絲等 (Bailey & Jeger 1992; Ullah *et al*. 2024)。然而，在以 *G. persicaria* 感染木瓜為模式的報告顯示，感染過程中沒有觀察到 *G. persicaria* 出現顯著的附著器 (Cruz-Lachica *et al*. 2018)。另外，根據本實驗室未顯示的資料，*G. persicaria* 於 28–32℃在殘體上至少可殘存 1 mo，因此推論本試驗中 *G. persicaria* 主要是以殘存形式存在於幼果表面，果實成熟後於高溫高濕等適合環境下伺機感染。

本研究對 *G. persicaria* 紅龍果分離株的真菌特性、病理性及發病生態做一初步的描述，尤其對發病生態的探討，有助瞭解最佳的防治時機與精準防治，希冀降低防治的成本與降低紅龍果濕腐病害引起的損失。

誌謝

感謝黃麟婷、林依佳、戴裕倫及詹月梅等助理的接力協助完成相關試驗。本研究承蒙 108 農科 -8.4.1- 農 -C1(7) 與 114 農科 -5.3.1- 農 -01(4) 等研究經費支持。

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Biological Characteristics and Pathogenicity of Pitaya Isolates of *Gilbertella persicaria*

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Abstract

Lin, C. P., M. C. Hsu, J. N. Tsai, J. W. Fan, and J. H. Huang. 2025. Biological characteristics and pathogenicity of pitaya isolates of *Gilbertella persicaria*. J. Taiwan Agric. Res. 74(4):497–513.

Wet rot of pitaya, caused by *Gilbertella persicaria*, is a significant disease affecting pitaya production in Taiwan. However, the biological characteristics and pathogenicity of the pathogen remain unknown. From 2009 to 2024, diseased pitaya tissues showing symptoms of wet rot were collected from 90 field sites across 23 townships in 11 counties, as well as from one local market in Taiwan. A total of 212 isolates of *G. persicaria* were obtained from pitaya, all of which belonged to the minus (-) mating type. Additionally, one isolate of *G. persicaria* from a diseased strawberry and another from a peach were both identified as the plus (+) mating type. These results indicate that both mating types of *G. persicaria* are present in Taiwan. To further investigate host-related differences among *G. persicaria* isolates, morphological, pathogenicity, and molecular phylogenetic analyses were conducted. The results showed that: (1) Morphologically, the number of sporangial wall segments in pitaya isolates was significantly higher than that of the type strain, by an average of 2.1–2.6 segments; (2) In pathogenicity assays, only pitaya isolates were able to induce typical wet rot symptoms on pitaya fruits. The results indicated that symptom type and lesion size were associated with the host origin of *G. persicaria* isolates, but not with their mating type; (3) In phylogenetic analysis, the phylogenetic tree based on concatenated sequences of internal transcribed spacer (ITS), actin, and 28S rDNA large subunit revealed that pitaya isolates formed a distinct monophyletic clade. Based on morphology, pathogenicity, and phylogenetic analyses, the pitaya isolates of *G. persicaria* were different from the type strain. Environmental condition assays indicated that optimal temperature and relative humidity range for disease development are 28–36℃ and above 80%. No disease symptoms developed at temperatures below 16℃ or above 40℃. Further inoculation trials were conducted on pitaya fruits at various developmental stages, followed by postharvest incubation at 25–32℃ to observe disease progression. These results demonstrated that fruits exhibited the most severe disease by postharvest inoculation, indicating that *G. persicaria* primarily infects pitaya during the fruit maturation stage, particularly at harvest.

Key words: Sporangial wall segment, Mating type, Pathogenicity, Phylogeny, Infection timing.

Received: June 4, 2025; Accepted: October 1, 2025.
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台灣農業研究 (J. Taiwan Agric. Res.) 74(4):515–524 (2025)
https://doi.org/10.6156/JTAR.202512_74(4).0009

研究報告

提高茄子種原發芽率初探

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摘要

夏奇鋕、曾馨儀、蔡挹恆、蔡嫺婷、莊淨、游舜期。2025。提高茄子種原發芽率初探。台灣農業研究 74(4):515–524。

茄子 (*Solanum melongena* L.) 為重要蔬菜亦為國家作物種原庫選定之核心種原，本研究以種原庫發芽率 (1%) 低下之茄子種原進行紙上法播種，比較浸種時間 (8、16 及 24 h)、溫湯處理 (55℃，15 min) 及培養溫度 (27℃恆溫或 30℃，16 h/20℃，8 h 變溫) 對發芽之影響，結果顯示 16 h 浸種可加速發芽，配合變溫培養在播種 2 wk 後之發芽率最高為 4.0%。將上述播種 2 wk 後未發芽之種子移至含有 0–200 mg L^{-1} GA_3 之 1/2 倍濃度 MS 培養基 (Murashige and Skoog medium) 中，並維持其原培養溫度條件，結果顯示在未添加 GA_3 之 1/2 MS 培養基中以恆溫培養 3 wk 後發芽率達 12.5%，而在添加 100 mg L^{-1} GA_3 培養基配合變溫培養處理中發芽率可達 25.0%。接續測試 0、1/2、1 倍濃度 MS 基礎鹽類作為培養液與添加 0、50 mg L^{-1} GA_3 之組合處理對種子發芽的影響，結果顯示 MS 與 GA_3 兩處理對發芽 (定義為胚根突出 2 mm) 的影響並不顯著，但 1/2 MS 或 50 mg L^{-1} GA_3 單獨處理皆有助發芽種子上胚軸芽體的生長，對後續小苗生長發育具有提升效果。

關鍵詞：茄子、種子、發芽、激勃素、MS 培養基。

前言

種原是建構育種工作的基礎要素，但種子壽命會受到物種、種子品質及保存條件等諸多因素影響，並且隨儲藏時間拉長而逐漸降低 (Desheva 2016; Yamasaki *et al*. 2020)。國家作物種原庫依種子的保存溫度與使用目的分為短、中及長期庫，其中又以中期庫 (1℃保存) 為最大宗之種原保存場所，提供種原交換、繁殖及評估之用。中期庫種子的保存時間一般為 10–20 年，為確保種子能維持一定之發芽率，且在種子發芽率大幅下降前進行種原更新工作 (Yamasaki *et al*. 2020)。更新程序為自中期庫房中取出種子進行發芽、植株栽培、植株成熟後收取種子及種子處理後重新入庫儲藏。上述種原更新工作的每一步驟皆有其重要性，但實務上最常遭遇的問題就是經過長期儲藏的種子發芽率偏低與發芽不整齊，增加了更新工作的困難度。究其原因可能為長期儲藏後種子老化、品質不佳或保存不當等，但亦有可能為種皮太厚導致吸水性不佳、種子休眠或發芽條件不適當等因素所造成 (Lamichhane *et al*. 2018)。種原庫一般會依種子之大小採用紙間法或紙上法進行發芽測試，但上述兩發芽法僅提供種子發芽所需水分，對於發芽率低下之重要種原而言，還需要開發更多提升發芽率之處理，以確保種原更新工作的進行。

茄子 (*Solanum melongena* L.) 為重要之茄科蔬菜，亦為國家作物種原庫選定之重要核心

投稿日期：2025 年 6 月 23 日；接受日期：2025 年 9 月 11 日。
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種原，茄子屬小粒種子但具堅硬且厚之種皮，種子之透水與透氣性較差，加上種子具休眠性，除造成茄子發芽率低外，並伴隨有發芽緩慢與不整齊的問題。本研究以國家作物種原庫中期庫中保存之茄子種原為研究材料，針對以紙上或紙間發芽法率偏低之茄子種原，除測試各種提高種子發芽率的方法外，並探討利用組織培養方法提高茄子種子發芽率的可行性，期望建立一套提高茄子種子發芽率的處理方法，確保珍貴茄子種原的永續利用。

材料與方法

茄子材料：種原庫暫時碼 2023A00487 號，該茄子種原以紙間法播種 (50 粒/重複，共 2 重複) 3 wk 後之發芽率為 1.0 ± 1.4%。

種子發芽流程：種子表面以酒精快速浸泡 30–60 s 後，移入 0.6% 次氯酸鈉水溶液以 140 rpm 震盪 18 min，再以無菌水以相同震盪轉速漂洗 3 次，每次 5 min。將滅菌後之種子置入無菌水中，於 27℃黑暗環境中以 80 rpm 震盪浸種 16 h。將完成浸種之種子以紙上法播種，流程為於 9 cm 玻璃培養皿中置入 2 張 1 號濾紙 (Advantec, Tokyo, Japan) 並以 3.5 mL 無菌水潤濕，將 25 粒種子置於濾紙上，以保鮮膜封口培養皿後放入保鮮盒後加蓋，置於溫度設定為 30℃ (16 h)/20℃ (8 h) 生長箱中進行暗培養。種子發芽之定義為胚根突破種皮 2 mm 以上，播種後每週調查發芽率 (發芽種子數/播種種子數) × 100%；發芽速度為累加 (每日發芽種子數/計算當日之天數)。下列試驗除另行描述之處理條件外，皆依上述種子發芽流程進行。

發芽小苗種植與植株生長調查：種子發芽後將小苗移植至 104 格穴盤，栽植 1 mo 後移植至 9 cm 盆，於 9 cm 盆種植 1.5 mo 後移植至 18 cm 盆，種植 3 mo 後移植至 27 cm 盆。介質配方均為培養土 (BVB Substrates, De Lier, The Netherlands)：珍珠石 (南海 3 號，南海園藝有限公司，臺灣新北市)：蛭石 (南海 3 號，南海園藝有限公司，臺灣新北市) = 3 : 1 : 1 (v/v/v)。於播種後 6 mo 後以捲尺測量株高與葉長，並於 1 mo 後再測量 1 次。

試驗 1

溫湯處理、浸種時間與培養溫度試驗：將種子以次氯酸水溶液消毒後，在無菌水第 3 次清洗時以 55℃或常溫 (對照組) 無菌水以磁石攪拌 15 min，完成後於 27℃黑暗環境中以無菌水浸種，並以 80 rpm 分別震盪 8、16 或 24 h，其中 24 h 浸種處理於浸泡 16 h 後換水 1 次。完成浸種之種子以紙上法播種，分別置於 27℃恆溫與 30℃ (16 h)/20℃ (8 h) 變溫培養，其他步驟同上述種子發芽流程。每一培養皿播種 25 粒種子為 1 重複，每一處理共 3 重複。每日調查種子發芽情形，調查期間為 2 wk。

試驗 2

MS 培養基與 GA_3 濃度試驗：將上述試驗 1 中恆溫或變溫處理培養 2 wk 後未發芽種子平均分配至含不同濃度 GA_3 (gibberellic acid, Sigma, USA) 培養基中，並以原來相同之溫度條件培養。培養基組成為含有 30 g L^{-1} 蔗糖與 8.5 g L^{-1} 洋菜之 1/2 倍 MS 基礎鹽類配方 (Murashige and Skoog Basal Medium with Vitamins, No. M519, PhytoTech Labs, Lenexa, KS, USA)，上述組成以下簡稱 1/2 MS 發芽培養基，並分別添加 0、25、50、100 及 200 mg L^{-1} GA_3 共 5 種濃度。每個 9 cm 培養皿中裝 25 mL 培養基，每皿接種 15 粒種子為 1 重複，每一處理共 6 重複。每週調查種子發芽情形，調查期間為 4 wk。

試驗 3

MS 基本鹽類濃度與 GA_3 濃度組合試驗：將浸種 16 h 後之種子以紙上法播種，每一培養皿加入 3.5 mL 培養液，培養液組成分別為 0、1/2 及 1 倍濃度之 MS 鹽類配方與 0、50 mg L^{-1} GA_3 組合之 6 個處理，每皿播種 30 粒種子為 1 重複，每一處理共 3 重複。

統計分析

本研究採用完全逢機設計 (completely randomized design; CRD) 進行試驗，試驗所得資料若為百分率，則先經角度轉換後再進行分析。經 SAS 套裝軟體 7.1 套裝統計分析軟體進行變方分析 (analysis of variance; ANOVA)，若處理間差異顯著 ($P < 0.05$)，則以最小顯著差異性測

驗 (least significant difference test; LSD test) 比較各處理平均值間之差異。

結果

試驗 1

溫湯處理、浸種時間與培養溫度試驗：本試驗探討溫湯處理、浸種時間及培養溫度 3 種處理對茄子種子發芽的影響，結果顯示種子在播種 2 wk 後，以浸種時間明顯影響發芽率 ($P < 0.01$) 與發芽速度 ($P < 0.05$)，兩兩因子或 3 個因子間的交感作用皆無顯著影響 (表 1)。從個別處理來看，以未經溫湯處理與浸種 16 h 並變溫培養之種子有最高發芽率為 4.0%。發芽速度則以經溫湯處理與浸種 16 h 並變溫培養種子之 0.59 粒/日最高。3 種處理中僅浸種時間對發芽率與發芽速度具顯著影響，浸種時間太短 (8 h) 或過長 (24 h)，發芽率皆降低，適當之浸種時間 (16 h) 配合變溫培養具有提高發芽速度與發芽率的效果。整體而言，種子發芽率仍偏低 (0.0–4.0%) 且發芽速度緩慢 (0.00–0.59 粒/日)。

試驗 2

1/2 MS 培養基與 GA_3 濃度試驗：因試驗 1 中以紙上法進行發芽之種子發芽率整體偏低 (最高為 4%)，因此將試驗 1 中恆溫或變溫處理培養 2 wk 後仍未發芽之種子分別合併後，平均分種於添加 0–200 mg L^{-1} 不同濃度 GA_3 之 1/2 MS 發芽培養基中，並以原培養溫度條件繼續培養，結果顯示在培養 1 wk 後，10 個組合處理中除低濃度 GA_3 (0 與 25 mg L^{-1}) 配

表 1. 茄子種原以溫湯、浸種時間及不同培養溫度處理之發芽情形。

Table 1. Germination of eggplant seeds treated with hot water dipping, soaking duration and culture temperature treatments.

Hot water	Soaking (h)	Culturing temperature	Germination rate (%) after weeks of sowing		Speed of germination[z] (No. seed d^{-1})
			1st	2nd	
No	8	constant[y]	0.0 ± 0.0 a[x]	0.0 ± 0.0 b	0.00 ± 0.00 a
No	8	variable	0.0 ± 0.0 a	0.0 ± 0.0 b	0.00 ± 0.00 a
Yes	8	constant	0.0 ± 0.0 a	0.0 ± 0.0 b	0.00 ± 0.00 a
Yes	8	variable	1.3 ± 2.3 a	2.7 ± 2.3 ab	0.46 ± 0.49 a
No	16	constant	0.0 ± 0.0 a	2.7 ± 2.3 ab	0.29 ± 0.27 a
No	16	variable	1.3 ± 2.3 a	4.0 ± 4.0 a	0.54 ± 0.67 a
Yes	16	constant	0.0 ± 0.0 a	1.3 ± 2.3 ab	0.18 ± 0.31 a
Yes	16	variable	2.7 ± 4.6 a	2.7 ± 4.6 ab	0.59 ± 1.00 a
No	24	constant	0.0 ± 0.0 a	0.0 ± 0.0 b	0.00 ± 0.00 a
No	24	variable	0.0 ± 0.0 a	0.0 ± 0.0 b	0.00 ± 0.00 a
Yes	24	constant	0.0 ± 0.0 a	0.0 ± 0.0 b	0.00 ± 0.00 a
Yes	24	variable	0.0 ± 0.0 a	0.0 ± 0.0 b	0.00 ± 0.00 a
Soaking duration (S)			ns[w]	**	*
Hot water (H)			ns	ns	ns
Culturing temp. (T)			ns	ns	ns
S × H, S × T, H × T, S × H × T			ns	ns	ns

[z] Speed of germination was summation of no. germinated seeds/days for germination.
[y] Constant temperature treatment was 27℃. Variable temperature treatment was 30℃ (16 h) /20℃ (8 h).
[x] Each data was mean ± *SD* of 3 replications; each replicate contained 25 seeds. Percentage data were logarithmic transformed prior to analysis. Means ± *SD* within each column with different letter are significantly different by least significant difference test (LSD test) at 5% level.
[w] Significance was determined by analysis of variance (ANOVA). ns, *, ** indicated non-significant, significant at 5% or 1% level, respectively.

合恆溫培養仍未有種子發芽外，其他各處理 (變溫培養或濃度高於 25 mg L^{-1} GA_3 處理) 皆有種子發芽，發芽率 2.1–9.4%，發芽率與試驗 1 相較明顯增加，顯示試驗 2 使用之 1/2 MS 發芽培養基與試驗 1 之無菌水相較，有助提高發芽率 (表 2)。因部分種子於 1/2 MS 發芽培養基中遭到汙染，導致某些處理之重複數減少，其中 0 mg L^{-1} GA_3、恆溫培養組合處理僅剩 1 重複，無法進行處理間之比較，因此僅就濃度 25–200 mg L^{-1} GA_3 處理進行 ANOVA 分析，結果顯示 GA_3 與培養溫度以及兩因子間的交感作用，在 1/2 MS 發芽培養基中的前 3 wk 皆顯示影響不顯著，但培養 4 wk 後 GA_3 與培養溫度兩因子皆顯示具有顯著影響 ($P < 0.05$)，但兩因子間的交感仍為不顯著。比較 GA_3 濃度處理影響，結果顯示 100 mg L^{-1} GA_3 處理在培養 3 wk 後，發芽率較其他 GA_3 濃度處理為高，其中又以變溫組合處理最高，在培養期間 (4 wk) 發芽率可持續增加至 31.3%。比較恆溫與變溫 2 種溫度處理，顯示恆溫處理在培養 2 wk 後，在 0–200 mg L^{-1} GA_3 處理之發芽率皆未再增加，但在變溫處理則顯示 100–200 mg L^{-1} GA_3 處理在 4 wk 培養期間發芽率持續增加 (表 2)。綜上結果推測 1/2 MS 發芽培養基、變溫培養與適當濃度之 GA_3 (100 mg L^{-1}) 皆有助提高種子發芽率。

上述處理之發芽小苗移至穴盤種植 1 mo 後更換至 9 cm 盆，觀察植株生長情形，由於試驗 2 部分處理有種子汙染問題，加上某些處理之發芽數偏低，導致特定處理之植株數太少或不等，難以進行統計分析，但植株生長調查主要在了解 GA_3 處理對於後續植株生長之影響 (表 3)。結果顯示在 9 cm 盆階段可觀察到部分源自 25–200 mg L^{-1} GA_3 處理小苗較源自 0 mg L^{-1} GA_3 處理 (對照組) 之小苗株高為長，其中又以源自 200 mg L^{-1} GA_3 處理之小苗其子葉與本葉間之莖長明顯較其他處理之幼苗為長 (圖 1)，顯示種子以較高濃度 GA_3 處理後對種子苗之莖長發育有促進作用；小苗在栽培 6–7 mo 後調查 5 種濃度 GA_3 處理植株之株高與葉長，結果顯示以 100–200 mg L^{-1} GA_3 處理較其他處理為矮；葉長方面，小苗栽培 6

表 2. 以紙上法播種 2 wk 後未發芽之茄子種子續以添加不同 GA_3 濃度之 1/2 MS 培養基培養 4 wk 之發芽情形。

Table 2. Germination rate of the ungerminated eggplant seeds[z] which were 2 wk after sowing using the paper method, and then followed by subculturing into 1/2 MS medium[y] with various GA_3 concentrations for 4 wk[z].

		Germination rate (%) after weeks of culturing in medium[z]			
GA_3 (mg L^{-1})	Culturing temperature[y]	$2^x + 1^{st}$	$2 + 2^{nd}$	$2 + 3^{rd}$	$2 + 4^{th}$
0	Constant	0.0 ± 0.0	12.5 ± 0.0	12.5 ± 0.0	12.5 ± 0.0
0	Variable	4.7 ± 9.4	7.8 ± 7.9	7.8 ± 7.9	7.8 ± 7.9
25	Constant	0.0 ± 0.0 a	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b
25	Variable	2.4 ± 3.3 a	3.7 ± 5.6 b	4.9 ± 8.1 b	4.9 ± 8.1 b
50	Constant	2.1 ± 3.2 a	3.1 ± 5.2 b	3.1 ± 5.2 b	3.1 ± 5.2 b
50	Variable	3.1 ± 3.6 a	6.3 ± 7.2 ab	7.8 ± 9.4 b	7.8 ± 9.4 b
100	Constant	6.3 ± 6.3 a	10.4 ± 9.5 ab	10.4 ± 9.5 ab	10.4 ± 9.5 ab
100	Variable	9.4 ± 13.3 a	18.8 ± 8.8 a	25.0 ± 8.8 a	31.3 ± 8.8 a
200	Constant	3.1 ± 6.3 a	3.1 ± 6.3 b	3.1 ± 6.3 b	3.1 ± 6.3 b
200	Variable	3.2 ± 3.7 a	4.8 ± 6.0 ab	4.8 ± 6.0 b	8.0 ± 9.5 b
GA_3 (G)		ns[w]	ns	ns	*
Culturing temperature (T)		ns	ns	ns	*
G × T		ns	ns	ns	ns

[z] Medium containing 1/2 MS salts, 30 g L^{-1} sucrose and 8.5 g L^{-1} agar.
[y] Culture temperature treatments were the same as the first seed sowing experiment.
[x] Eggplant seeds were the ungerminated seeds derived from the first seed sowing experiment after 2-wk-culturing.
[w] Significance was determined by analysis of variance (ANOVA). ns, *, ** indicated non-significant, significant at 5% or 1% level, respectively.

mo 後在 14.8–17.8 cm 間，栽培 7 mo 後葉長在 17.0–22.1 cm 之間，GA_3 不同濃度處理間並未有明顯差異 (圖 2)。上述結果顯示高濃度 100–200 mg L^{-1} GA_3 處理在小苗階段有促進節間伸長之效果，但對植株長期生長後之株高可能有負面影響。

試驗 3

MS 濃度與 GA_3 濃度組合試驗：由試驗 2 結果顯示 1/2 MS 鹽類配方作為培養液有助提高發芽率，本研究進一步測試 0、1/2 及 1 倍濃度 MS 鹽類配方作為培養液並分別添加 0 與 50 mg L^{-1} GA_3 共 6 種處理對種子發芽之影響。結果顯示 MS 濃度與 GA_3 兩因子與兩因子間

表 3. 以不同 GA_3 濃度處理之茄子種子之發芽小苗後續植株生長情形。

Table 3. Plant growth of the germinated seedlings of eggplant derived from seeds treated with different GA_3 concentrations.

	Plant height (cm)		Leaf length (cm)	
GA_3 (mg L^{-1})	6 mo	7 mo	6 mo	7 mo
0	72.2 ± 14.0	87.4 ± 16.1	14.8 ± 1.4	20.3 ± 2.9
25	79.6 ± 2.5	89.9 ± 6.4	17.8 ± 3.3	19.0 ± 3.0
50	75.5 ± 0.0	87.2 ± 0.0	15.5 ± 0.0	17.0 ± 0.0
100	65.4 ± 10.2	75.7 ± 7.6	16.5 ± 2.8	22.1 ± 2.3
200	70.3 ± 0.0	71.3 ± 0.0	15.6 ± 0.0	20.0 ± 0.0

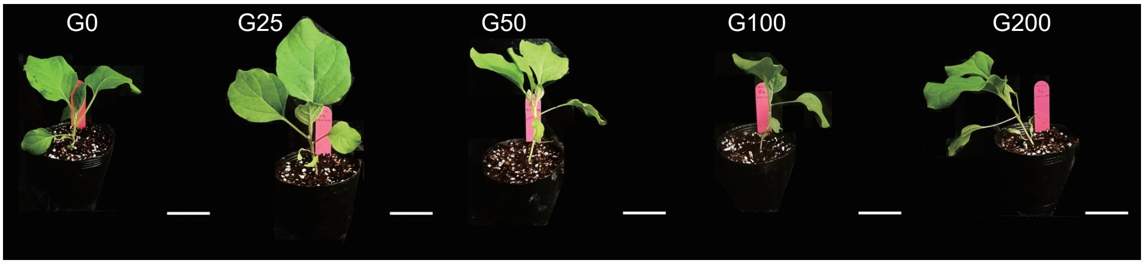

圖 1. 以不同濃度 GA_3 (mg L^{-1}) 處理之發芽種子移種於溫室栽植 1 mo 之生長情形。

Fig. 1. The growth of germinated seeds treated with GA_3 at different concentrations (mg L^{-1}), after being cultivated in the greenhouse for 1 mo. Bar = 5 cm.

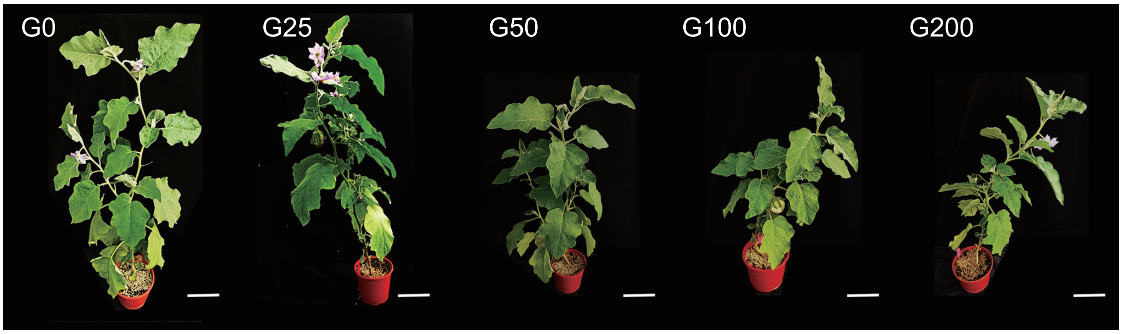

圖 2. 以 5 種濃度 GA_3 (mg L^{-1}) 培養基處理之茄子種子在發芽 7 mo 後植株生長情形。

Fig. 2. Plant growth of eggplant derived from seeds treated with five concentrations of GA_3 (mg L^{-1}) in germination medium and then transplanted for 7 mo after germination. Bar = 20 cm.

的交感作用，對第一粒種子發芽所需時間的影響皆不顯著，6 個組合處理之第一粒種子發芽所需時間在 6.0–7.3 d 間，MS 濃度與 GA_3 濃度處理未明顯縮短發芽時間 (表 4)。MS 濃度與 GA_3 濃度兩因子與兩因子間的交感作用在 1–7 wk 培養期間皆顯示對種子發芽率的影響不顯著，在相同培養時間下各處理間的發芽率均顯示無明顯差異。以播種後第 3 週來看 MS 濃度的影響，發芽率最低為無菌水處理，其次為 1.0 倍 MS，以 0.5 倍 MS 為最佳；以 GA_3 處理的影響來看，無菌水添加 50 mg L^{-1} GA_3 有助提高發芽率，在 1/2 MS 添加 GA_3 對發芽率的影響差異不大，但在 1.0 倍 MS 處理中添加 GA_3 則導致發芽率降低，但此一情形隨著培養時間增加逐漸改善。因為各處理內之標準偏差較高，上述處理間的比較在統計上並未有明顯差異，但各處理之平均值的變動仍可作為改進之參考。

由於種子發芽率調查係根據胚根突出種皮 2 mm 長度作為判斷依據，但對發芽率低下的種子而言，在胚根長出後其地上部芽體的生長亦同樣面臨障礙。本試驗於種子播種 3 wk 後開始調查已發芽種子 (胚根突出種皮 2 mm) 其地上部芽體發育之情形 (表 5)。結果顯示 MS 處理在播種培養 3–7 wk 影響不顯著，但至第 8 週時則顯示影響顯著 ($P < 0.05$)。GA_3 處理在第 5–6 週時即顯示影響顯著 ($P < 0.05$)，兩因子之交感作用在培養期間皆顯示為不顯著。雖然 MS 濃度與 GA_3 處理在調查種子之發芽率影響不顯著 (表 4)，但持續觀察發根種子其地上部之生長，以成苗率表示於表 5，結果顯示適當之 MS 濃度 (0.5 倍) 以及 50 mg L^{-1} GA_3 添加可提高種子之成苗率，亦即 MS 與 GA_3 處理對種子發芽之影響主要在地上部芽體的生長，而非胚根的突出種皮。整體而言，以 0.5 倍 MS 無論有無添加 GA_3 處理皆有較高比例之種子發芽率及幼苗發育率，GA_3 對於無菌水或全量 MS 處理之種子地上部芽體生長具有提升之效果，但在統計上多未有明顯差異。

討論

本研究以種原庫發芽率 (1%) 低下之茄子種原為材料，探討提高發芽率之可行方法。在試驗 1 中測試溫湯處理、浸種時間與培養溫度 3 個處理，結果顯示僅浸種時間顯著提高發芽率與發芽速度，且其影響在播種後 2 wk 即快速顯現 (表 1)。浸種是最簡單、經濟且環境友善的種子處理，經過適當水溫與浸泡時間處理的種子逐漸恢復至較佳的水分狀態，種子內部各種生理機能得以活化，並對外界環境產生反應能力，從而提高種子的發芽率，對不同種的植物而言，如何達到種子最佳發芽狀態，浸種條件扮演重要之關鍵角色 (Dürr *et al*. 2015)。Adhikari *et al*. (2021) 將苦瓜種子浸種 6–48 h，種子含水率隨浸種時間延長而逐漸提高，以 48 h 浸種有最高之含水率，同時也具有最高發芽率與生長勢。在本研究中浸種使用 8、16 及 24 h，3 個浸種時間中以 16 h 之發芽率最高，推測浸種 8 h 有可能仍不足以軟化種皮促進水分的吸收，但浸種 24 h 則可能因浸泡時間過長而對種子產生不良影響，如缺氧、發霉或營養流失等不利發芽之情形。溫湯處理 (55℃，15 min) 在本研究中對種子發芽之直接影響雖不顯著，但結果亦顯示在浸種時間不足時 (如 8 h)，施用溫湯處理配合變溫處理對提升種子發芽率仍有幫助 (表 1)。

茄子為源自熱帶的作物，發芽溫度介於 20–30℃，最適發芽溫度在 24–29℃，因種子具休眠性，發芽緩慢不整齊，一般將其歸類為低發芽率物種 (Chen & Li 1996)。Botey *et al*. (2022) 以同屬熱帶之非洲茄子 (*S. aethiopicum*) 進行 15–35℃發芽溫度試驗，結果顯示以 20℃ (發芽率 74%) 或 25℃ (發芽率 76%) 最佳，低於 15℃或高於 30℃發芽率皆降低，但若以 30/20℃變溫處理配合 8 h 光照/16 h 暗處理，則有最高發芽率 (76–95%) 與最短發芽時間 (4–5 d)，顯示變溫處理有助種子發芽率提升。本研究之結果亦顯示變溫處理較 27℃恆溫培養有助提高發芽率，雖然在試驗 1 中培養 2 wk 時並未呈現顯著影響，但在試驗 2 中持續培養至第 6 週 (2 + 4 wk) 時，則顯示溫度處理對發芽率具顯著影響，表 2 中除 0 mg L^{-1} GA_3 外，其他 GA_3 濃度皆以變溫處理較恆溫處理有利於發芽，推測培養溫度較浸種影響為慢的原因，可能與 27℃恆溫培養亦為茄子之發芽適

表 4. 播種培養液中 MS 鹽類與 GA_3 濃度對茄子種子發芽之影響。

Table 4. Effects of the strength of MS salts and GA_3 concentration in sowing culture medium on germination of eggplant seeds.

MS strength	GA_3 (mg L^{-1})	Days of 1st seed germinated	Germination (%) after weeks of sowing						
			1st	2nd	3rd	4th	5th	6th	7th
0	0	6.3 ± 0.6 a[z]	4.4 ± 1.9 a	6.7 ± 5.8 a	7.8 ± 5.1 a	8.9 ± 5.1 a	12.2 ± 5.1 a[z]	14.4 ± 6.9 a	15.6 ± 7.7 a
0	50	7.3 ± 2.3 a	6.7 ± 6.7 a	8.9 ± 3.8 a	11.1 ± 7.7 a	16.7 ± 3.3 a	22.2 ± 1.9 a	25.6 ± 1.9 a	25.6 ± 1.9 a
1/2	0	6.0 ± 0.0 a	8.9 ± 3.8 a	10.0 ± 3.3 a	16.7 ± 6.7 a	20.0 ± 8.8 a	24.4 ± 6.9 a	28.9 ± 10.7 a	31.1 ± 10.2 a
1/2	50	6.3 ± 0.6 a	10.0 ± 6.7 a	11.1 ± 8.3 a	15.6 ± 10.2 a	17.8 ± 13.9 a	24.4 ± 13.9 a	25.6 ± 12.6 a	27.8 ± 13.9 a
1	0	6.0 ± 0.0 a	7.8 ± 1.9 a	14.4 ± 8.4 a	16.7 ± 8.8 a	16.7 ± 8.9 a	22.2 ± 12.6 a	22.2 ± 12.6 a	23.3 ± 12.0 a
1	50	6.0 ± 0.0 a	4.4 ± 1.9 a	7.8 ± 1.9 a	11.1 ± 1.9 a	17.8 ± 1.9 a	23.3 ± 3.3 a	23.3 ± 3.3 a	24.4 ± 1.9 a
MS	ns[y]	ns	ns	ns	ns	ns	ns	ns	ns
GA_3	ns	ns	ns	ns	ns	ns	ns	ns	ns
MS × GA_3	ns	ns	ns	ns	ns	ns	ns	ns	ns

[z] Each data was mean ± *SD* of 3 replications; each replicate contained 30 seeds. Percentage data were logarithmic transformed prior to analysis. Means ± *SD* within each column with different letters are significantly different by least significant difference test (LSD test) at 5% level.
[y] Significance was determined by analysis of variance (ANOVA), ns indicated non-significant at 5% level.

表 5. MS 鹽類濃度與 GA_3 濃度對已發芽茄子種子地上部形成之影響。

Table 5. Effects of the MS salt strength and GA_3 concentration on the shoot formation of germinated eggplant seeds.

Treatment		Shoots formation rate (%)					
MS salt (strength)	GA_3 (mg L^{-1})	3rd [z]	4th	5th	6th	7th	8th
0	0	1.1 ± 1.9 ab[y]	2.2 ± 3.8 ab	2.2 ± 3.8 b	4.4 ± 7.7 bc	4.4 ± 7.7 bc	5.6 ± 9.6 bc
0	50	3.3 ± 3.3 ab	8.9 ± 5.1 a	11.1 ± 8.4 a	13.3 ± 8.8 ab	13.3 ± 8.8 abc	14.4 ± 7.7 ab
1/2	0	5.6 ± 6.9 ab	8.9 ± 6.9 a	13.3 ± 3.3 a	15.6 ± 3.8 a	17.8 ± 3.8 a	17.8 ± 3.8 a
1/2	50	4.4 ± 1.9 a	7.8 ± 5.1 ab	11.1 ± 5.1 a	12.2 ± 5.1 ab	14.4 ± 6.9 ab	16.7 ± 3.3 a
1	0	0.0 ± 0.0 b	1.1 ± 1.9 b	2.2 ± 3.8 b	2.2 ± 3.8 c	3.3 ± 5.8 c	3.3 ± 5.8 c
1	50	2.2 ± 1.9 ab	4.4 ± 5.1 ab	8.9 ± 6.9 ab	8.9 ± 6.9 abc	8.9 ± 6.9 abc	8.9 ± 6.9 abc
MS		ns[x]	ns	ns	ns	ns	*
GA_3		ns	ns	*	*	ns	ns
MS × GA_3		ns	ns	ns	ns	ns	ns

[z] Weeks after sowing.
[y] Each data was mean ± *SD* of 3 replications; each replicate contained 30 seeds. Percentage data were logarithmic transformed prior to analysis. Means ± *SD* within each column with different letters are significantly different by least significant difference test (LSD test) at 5% level.
[x] Significance was determined by analysis of variance (ANOVA), ns and * indicated non-significant or significant at 5% level, respectively.

溫，因兩處理間的差異相對較小，可能需較長時間後方能呈現出兩者間之差異 (表 2)。

試驗 2 與試驗 1 最大之差異除 GA_3 外，為試驗 2 採用組織培養之培養基代替無菌水作為培養介質，在培養 2 wk 後，使用 1/2 MS 發芽培養基 2 wk 後之發芽率在 10.0–11.1% (表 4)，與表 1 中使用無菌水之發芽率 0–4% 相比，發芽率提高具有普遍性，且在未添加 GA_3 之 1/2 MS 發芽培養基 (恆溫培養) 培養 2 wk 之發芽率亦可達 12.5%，推測與使用 1/2 MS 發芽培養基有關 (表 2)。一般為提高種子發芽率常以含有氮、磷、鉀或微量元素之化學物質進行種子滲調處理 (Salles *et al*. 2019; Hosseinifarahi 2022)。推測本研究試驗 2 與 3 中使用之 1/2 MS 培養基中含有之各種鹽類與維生素提供種子相似於上述滲調化學物之效果；此外，種子發芽介質的物理性質亦為影響發芽之重要因子，介質之保水性、通氣、排水性及離子交換性皆為影響種子發芽之因子 (Lamichhane *et al*. 2018)，本研究中使用組織培養之洋菜培養基與紙上法使用之純水相較，推測前者具有更好的物理或化學基質 (matrix) 特性是較水更佳的發芽介質。

Gisbert *et al*. (2011) 為提高茄子 (*S. melongena*) 與茄子相關種原 *S. macrocarpon*、*S. aethiopicum* 及 *S. incanum* 之發芽率，將種子消毒後培養於培養基，發現以組織培養方式培養之種子發芽率明顯高於紙上法 (增加 2.44 倍)，而且若於培養基中添加 1 mg L^{-1} GA_3，除可提高發芽率 (2.95 倍) 外，並可縮短發芽時間。本研究在試驗2的1/2 MS發芽培養基 (0 mg L^{-1} GA_3) 中最高發芽率為 12.5% (表 2)，與本研究在試驗 1 中使用紙上法最佳之發芽率 4% 相較 (表 1)，增加 3.13 倍，而在 1/2MS 發芽培養基中添加 100 mg L^{-1} GA_3 培養基培養 3 wk 之最佳發芽率為 25.0%，增加 6.25 倍。種子老化是影響種子萌發的一個因素，隨著種子齡增加，種子發芽率隨之下降，Demir *et al*. (1994) 的研究顯示 GA_3 處理對老化種子之發芽有促進作用。Demir *et al*. (1994) 對儲藏 2 年與 5 年茄子種子以 10、50 mg L^{-1} GA_3 進行 6 h 浸泡處理，結果顯示 GA_3 處理對 5 年齡種子發芽率提升較 2 年齡種子更為明顯，但 GA_3 對後續地上部芽體、根生長及小苗乾種皆無影響。

GA_3 被認為在種子發芽過程中可誘導軟化胚乳之酵素合成，有利細胞伸長與小苗生長；在 Neto *et al*. (2017) 研究顯示茄子種子以 750 mg L^{-1} GA_3 經 12 h 浸泡處理在 30 d 後調查小苗株高，結果顯示處理組株高明顯較未施用 GA_3 之對照株為高，但在施用 45 d 後調查則不再有差異。在本研究中於 9 cm 盆階段可觀察到 200 mg L^{-1} GA_3 處理小苗之節間明顯較長 (圖 1)，此一結果與 Neto *et al*. (2017) 之觀察相似，但本研究在栽培 6–7 mo 後顯示以 100–200 mg L^{-1} GA_3 處理之株高較其他處理為矮 (表 3)，此一結果則以 Neto *et al*. (2017) 之觀察相異，推測本研究雖然使用之 GA_3 濃度較 Neto *et al*. (2017) 使用之濃度為低，但 Neto *et al*. (2017) GA_3 處理為 12 h，與本研究添加 GA_3 於培養基中處理時間為 4 wk 相較明顯較短有關。

為降低種子因消毒不完全導致汙染而影響發芽，試驗 3 以不含蔗糖與洋菜之 MS 培養液代替試驗 2 中之固態培養基，並分別添加 0 或 50 mg L^{-1} GA_3，結果顯示雖然 MS 與 GA_3 兩因子在 1–8 wk 期間對調查胚根突出之發芽率影響不顯著 (表 4)，但若持續觀察地上部芽體之生長，亦即以成苗率來看 (表 5)，則可觀察到 GA_3處理在培養第5–6週時呈現顯著影響 ($P < 0.05$)，MS 濃度的影響則在培養 8 wk 後呈現顯著影響 ($P < 0.05$)，顯示兩者皆對成苗率皆有顯著影響。以適當 MS 濃度 (0.5 倍) 培養時，無論有無添加 GA_3 成苗率皆較高，但若培養液不適當時 (0 或 1.0 倍 MS)，則 50 mg L^{-1} GA_3 添加對種子之地上部芽體之生長有促進效果，但在統計上多未有顯著差異。

綜合本研究在茄子種原發芽試驗之結果，顯示浸種是最快速提高種子發芽的方法，浸種時間以16 h效果最佳，於30℃ (16 h)/20℃ (8 h) 下使用添加蔗糖與洋菜之 1/2 MS 固態培養基 (1/2 MS 發芽培養基) 或只含 1/2 MS 基本鹽類之培養液皆可提高發芽率。利用組織培養方法可提供種子穩定之發芽環境，雖成本較高但對於發芽率偏低的珍貴種原而言，若無法順利進

行種原更新，同樣也要付出高成本。在本試驗中以紙間法發芽率為 1% 之種子，在經過一般物理方法 (溫湯與浸種) 提高發芽率無效後，再繼續移種 1/2MS 發芽培養基培養 3 wk 後，可獲得 25.0% 發芽率且仍持續發芽，應可作為挽救低發芽率珍貴種原之用。

誌謝

本研究承蒙農業部 113 農科–4.6.1–農–04 (3) 計畫經費支持，謹此致謝。

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Study on the Improvement of Seed Germination of Eggplant Germplasm

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Abstract

Hsia, C. N., H. Y. Tseng, Y. H. Tsai, W. T. Tsai, C. Chuang, and S. C. You. 2025. Study on the improvement of seed germination of eggplant germplasm. J. Taiwan Agric. Res. 74(4):515–524.

Eggplant (*Solanum melongena* L.) is an important vegetable and a core collection of the National Plant Genetic Resources Center in Taiwan. Seeds of eggplant with a low germination rate (1%) in the seed bank stock were used in this study. Effects of immersion duration (8, 16, and 24 h), hot water treatment (55℃ for 15 min), and culture temperature (27℃ for 24 h, and 30℃ for 16 h/20℃ for 8 h) on seed germination were investigated utilizing the paper germination method. Results showed that among the three factors, only immersion duration significantly increased the seed germination 2 wk after sowing, and the highest germination rate of 4.0% was obtained with immersion for 16 h with variable temperature culture (30℃/20℃, 16 h/8 h). The above-mentioned ungerminated seeds 2 wk after sowing were transplanted into a 1/2 MS medium (Murashige and Skoog medium) supplemented with 0–200 mg L^{-1} GA_3 and consistently cultured under the same temperature conditions, which were either constant 27℃ or variable temperature conditions. The results showed that 1/2 MS culture medium without GA_3 had a 12.5% germination rate after 3 wk of culture. Furthermore, an increased germination rate of 25.0% was achieved by combining 1/2 MS culture medium with 100 mg L^{-1} GA_3 and cultured in variable temperature conditions. Treatments in combination with MS basal salt concentration (0, 1/2, and 1 strength) and GA_3 concentration (0 and 50 mg L^{-1}) in the sowing culture solution were tested successively. Although the results showed that the two factors had no significant effect on seed germination (based on radicle protrusion 2 mm), enhanced shoot germination rates were observed on the treatments with either 1/2 MS or 50 mg L^{-1} GA_3 alone.

Key words: *Solanum melongena* L., Seed, Germination, Gibberellic acid, MS medium.

Received: June 23, 2025; Accepted: September 11, 2025.
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台灣農業研究 (J. Taiwan Agric. Res.) 74(4):525–539 (2025)
https://doi.org/10.6156/JTAR.202512_74(4).0010

Research Article

Effects of Orchard-Level Variation and Cultivation Method on the Functional Quality of Indian Jujube (*Ziziphus mauritiana*)

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Abstract

Hung, T. H., C. H. Chang, C. H. Chen, S. Y. Chen, M. C. Hsu, and J. H. Guo. 2025. Effects of orchard-level variation and cultivation method on the functional quality of Indian jujube (*Ziziphus mauritiana*). J. Taiwan Agric. Res. 74(4):525–539.

This study investigated the effects of cultivation method (organic vs. conventional) and orchard-level variation on the functional quality of Indian jujube (*Ziziphus mauritiana*) fruits. A total of 64 fruit samples were collected from four orchards in Taiwan- two organic and two conventional- and analyzed for their antioxidant capacity, total phenolic content, total flavonoid content, and vitamin C content. Although organically grown fruits exhibited slightly higher mean levels of antioxidant capacity, phenolic compounds, and flavonoid compounds, linear mixed-effects models indicated that the cultivation method had no statistically significant effect on any functional indicators. In contrast, orchard-level random effects accounted for a substantial proportion of the total variance, particularly for antioxidant capacity, total flavonoids, and vitamin C. Notable differences were observed between orchards within the same cultivation method, suggesting that localized environmental or management conditions may play a stronger role than cultivation system alone in shaping fruit phytochemical profiles. Measured soil nutrient profiles revealed significant differences among orchards, providing plausible explanatory cues for observed phytochemical variation. Pearson correlation analysis further revealed that antioxidant capacity was strongly associated with total phenolic and flavonoid content. In contrast, vitamin C showed no significant correlations with other traits, indicating that phenolic and flavonoid compounds are likely the primary contributors to antioxidant potential in Indian jujube fruits.

Key words: Indian jujube, Functional quality, Cultivation method, Orchard-level variation, Linear mixed-effects model.

Received: June 30, 2025; Accepted: September 12, 2025.

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INTRODUCTION

Ziziphus mauritiana Lam., commonly known as Indian jujube, is a member of the Rhamnaceae family and has a long history of cultivation in South Asia and East Africa (O'Brien *et al.* 2023). The species is widely cultivated across tropical and subtropical regions of Asia, including India, Pakistan, China, and Taiwan, where it is regarded as an important economic fruit crop (Koley *et al.* 2016; Duan & Chen 2022). Due to its adaptability to harsh environments and high nutritional value, Indian jujube is also valued in several North and East African countries, where it contributes to food security in resource-limited arid regions (Maruza *et al.* 2017; O'Brien *et al.* 2023).

In Taiwan, Indian jujube- locally known as "zaozi" or "mizao"- is a culturally significant fruit that is widely consumed during the Lunar New Year season. It is associated with prosperity and auspiciousness, making it a popular choice among consumers and a key source of seasonal income for farmers. The crop is cultivated extensively in southern Taiwan, particularly in Kaohsiung, Pingtung, and Tainan, covering approximately 1,700 ha (Agriculture Statistics Inquiry System, https://agrstat.moa.gov.tw/sdweb/public/inquiry/InquireAdvance.aspx). In addition to fresh consumption, Indian jujube is processed into a variety of value-added products, including candied jujube, dried jujube, and jujube juice, thereby enhancing its economic potential.

Indian jujube is known for its sweet flavor and high nutritional value, containing carbohydrates, proteins, dietary fiber, and essential minerals such as potassium, calcium, sodium, phosphorus and magnesium (Nyanga *et al.* 2013; Prakash *et al.* 2021). It is also a rich source of vitamins, including thiamine, riboflavin, niacin, and ascorbic acid (Prakash *et al.* 2021; Shady *et al.* 2022). Of particular interest are its polyphenolic and flavonoid constituents, which have been associated with antioxidant and health-promoting properties (Koley *et al.* 2016; Shady *et al.* 2022). Identified phenolic acids include caffeic acid, chlorogenic acid, ferulic acid, and p-coumaric acid, while flavonoids such as quercetin 3-O-glucoside, epicatechin, and naringenin are also present (Memon *et al.* 2013; Prakash *et al.* 2021; Khanam *et al.* 2025). Furthermore, Indian jujube has been reported to exhibit anti-inflammatory and antidiabetic effects in pharmacological studies (Sangeethapriya & Siddhuraju 2014; Khanam *et al.* 2025), and its high dietary fiber content may support digestive health (Sarkar *et al.* 2022).

With growing demand for food safety and health, organic fruit production has gained increasing attention. Organic agriculture emphasizes ecological sustainability by minimizing the use of synthetic pesticides and fertilizers, and it is often associated with the production of higher-quality and potentially more health-promoting fruits. Several studies have reported that organically grown fruits and vegetables exhibit elevated levels of bioactive compounds, including polyphenols and vitamin C, compared to conventionally grown counterparts (Brandt *et al.* 2011; Średnicka-Tober *et al.* 2020). While the nutritional profile of Indian jujube is known to be influenced by genotype and cultivation environment (Koley *et al.* 2016; Meena *et al.* 2023), a notable lack of research remains in comparing the functional composition of organically and conventionally grown Indian jujube, particularly in subtropical regions like Taiwan. Moreover, orchard-specific environmental and management conditions, such as microclimate, soil fertility, and tree vigor, may also modulate fruit phytochemical expression but are rarely considered in comparative studies (Stracke *et al.* 2009; Rocchetti *et al.* 2022).

This study aims to evaluate how different cultivation systems (organic vs. conventional) and orchard-level variation (such as soil physicochemical properties) influence the functional quality of Indian jujube fruits cultivated in Taiwan. Here, functional quality refers to phytochemical attributes with antioxidant relevance, specifically total phenolic content, total flavonoid content, vitamin C concentration, and overall antioxidant capacity. Through biochemical analyses and linear mixed-effects modeling,

this research provides an integrative perspective on how farming practices and site-specific conditions shape the phytochemical profiles of Indian jujube. The results are expected to inform both scientific understanding and support evidence-based strategies for enhancing the nutritional value of this culturally and economically significant fruit crop.

MATERIALS AND METHODS

Plant materials

This study was conducted at the Kaohsiung Yanchao Long-Term Ecological Research Station in southern Taiwan, which features a tropical monsoon climate (annual rainfall ~1,800 mm, mostly from May to October; average temperature 23–25℃). Fruit samples were collected from four independent orchard blocks, each ≥ 0.2 ha in area, planted with the same variety (*Z. mauritiana* 'Mizao') under either organic or conventional cultivation systems. Detailed orchard information is provided in Appendix 1, and orchard images and locations are shown in Appendix 2. These orchards were designated as follows:

Organic orchards (orchards A and B) are located in Guanshui and Antong, respectively, within the Yanchao District. The orchards are approximately 4 km apart and have been managed organically for over 20 years. The organic production process is certified by Blue Magpie Certifications (Certification No. 1-017-224006, Taipei, Taiwan) and adheres to the Shumei Natural Agriculture philosophy, which prohibits the use of pesticides, chemical fertilizers, and organic manures. Trees are grown under 16-mesh net house conditions with single-layer horizontal trellising, spaced 6 m × 6 m, and irrigated using well water.

Conventional orchards (Orchards C and D) are located in the Guanshui section of Yanchao District. The orchards occupy adjacent cadastral parcels and have been managed under conventional cultivation practices for over 10 years. They use a 6 m × 6 m planting grid and cultivate under 16-mesh nylon netting. Fertilization involves the annual application of NPK fertilizers a 4:2:5 ratio, with 1,500 g applied per tree. Pest and disease control involves the timely application of registered agrochemicals: 10% fenpyroximate suspension concentrate (SC) at 2,000× dilution (1,000 L ha^{-1}) for mite control; 40% kresoxim-methyl wettable powder (WP) at 1,000× dilution (2,000 L ha^{-1}) during the fruiting stage for powdery mildew control; and 23% azoxystrobin SC at 3,000× dilution (2,000 L ha^{-1}) during the fruiting stage for anthracnose control.

Across all orchards, only trees aged ≥ 10 years showing healthy growth without visible pest or disease damage were selected. For each orchard, a total of 16 fruits per orchard were randomly harvested from mid-canopy positions at commercial maturity (~80% ripeness), as identified by yellow-green skin with slight softening (Fig. 1) (Guo *et al*. 2022). The fruits were stored at ambient temperature for subsequent phytochemical and antioxidant analyses.

Sample preparation for phytochemical analyses

Approximately 50 g of fresh Indian jujube fruit was homogenized with 200 mL of distilled water using a commercial blender (JVE-1758W, CookPower, New Taipei, Taiwan) to produce a uniform juice. A 20 mL aliquot of the juice was then mixed with 20 mL of methanol and subjected to ultrasonic extraction. Ultrasonic extraction was conducted using an ultrasonic bath (DC400H, DELTA, New Taipei, Taiwan) operating at 40 kHz and 400 W for 20 min at room temperature. After extraction, the mixture was

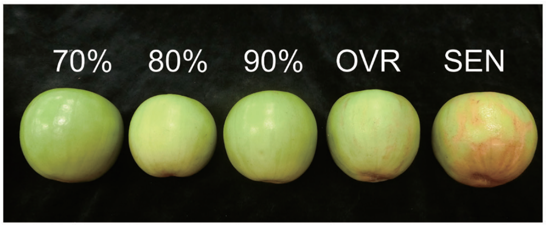


Fig. 1. Indian jujube fruit maturity stages. From left to right: 70% mature, 80% mature (commercial standard), 90% mature, overripe (OVR), and senescence (SEN).

centrifuged at 2,400 × *g* for 10 min using a centrifuge (Model 4000, KUBOTA, Osaka, Japan). The resulting supernatant was collected and used to determine the antioxidant capacity, total phenolic content, and total flavonoid content.

Antioxidant capacity

Antioxidant capacity was evaluated using the ABTS radical cation decolorization assay, as described by Thaipong *et al*. (2006), with slight modifications. The $ABTS^{\bullet+}$ solution was prepared by reacting 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (A3219, Sigma-Aldrich, Saint Louis, MO, USA) with 30 mM ammonium persulfate (101200, Merck, Darmstadt, Germany) at a 10:1 (v:v) ratio and allowing the mixture to stand in the dark at room temperature for 1.5 h to generate the radical cation. Before the assay, the $ABTS^{\bullet+}$ solution was diluted with distilled water to obtain an absorbance of 1.0–1.5 at 734 nm.

For the measurement, 180 µL of diluted $ABTS^{\bullet+}$ solution was mixed with 20 µL of the sample extract or distilled water (blank) in a 96-well microplate. The absorbance was measured at 734 nm at room temperature using a microplate reader (SpectraMax250, Molecular Devices, San Jose, CA, USA). Trolox (0–800 µM) was used to generate the standard calibration curve. Results were expressed as Trolox equivalent antioxidant capacity per gram of fresh weight (mg TE g^{-1}).

Total phenolic content: The total phenolic content of the jujube extracts was determined using the Folin-Ciocalteu colorimetric method, as described by Noreen *et al*. (2017), with minor modifications. Briefly, 50 µL of sample extract was mixed with 50 µL of 0.5N Folin-Ciocalteu reagent (109001, Merck, Darmstadt, Germany) in a 96-well microplate. After 5 min, 100 µL of a 20% sodium carbonate solution (31432, Honeywell, Offenbach am Main, Germany) was added, and the mixture was incubated at room temperature in the dark for 5 min. The absorbance was measured at 750 nm using a microplate reader. Gallic acid (410862500, Acros Organics, Geel, Belgium) was used as a standard to construct the calibration curve (0–100 µg mL^{-1}), and results were expressed as milligrams of gallic acid equivalents per gram of fresh weight (mg GAE g^{-1}).

Total flavonoid content: The total flavonoid content of the jujube extracts was determined following the colorimetric method described by Do *et al*. (2014), with slight modifications. Briefly, 100 µL of the sample extract was mixed with 25 µL of 2% sodium nitrite ($NaNO_2$) solution and allowed to react for 6 min at room temperature. Then, 30 µL of 10% aluminum chloride ($AlCl_3$) solution was added, followed by incubation for another 6 min. Subsequently, 200 µL of 1 M sodium hydroxide (NaOH) was added, and the final volume was adjusted to 1 mL with distilled water. The absorbance was measured at 510 nm using a microplate reader. Rutin was used to generate the standard calibration curve (0–500 µg mL^{-1}), and results were expressed as milligrams of rutin equivalents per gram of fresh weight (mg RUE g^{-1}).

Vitamin C content

The vitamin C content of fresh jujube fruits was determined using an RQflex® 20 reflectometer (Merck, Darmstadt, Germany) in conjunction with the corresponding Ascorbic Acid Test strips (Product No. 116981, Merck, Darmstadt, Germany), following the manufacturer's instructions. Fresh fruits were finely grated using a stainless-steel grater to obtain juice. A small volume of the juice was applied directly onto the reaction zone of the test strip, which was then inserted into the reflectometer. The instrument automatically recorded the change in reflectance intensity and calculated the vitamin C content by referencing an internal calibration curve. Results were expressed as milligrams of vitamin C per 100 mL of juice (mg 100 mL^{-1}).

Soil fertility analysis

Soil samples were collected before jujube fruit harvest (December) from each orchard. For each orchard, 5 random sampling points were selected. At each point, soil was taken from 3 depths: surface (0–15 cm), subsurface (15–30

cm), and bottom (30–45 cm). The samples from the same orchard were thoroughly mixed and air-dried for 5–7 d at ambient temperature. After drying, the soil was gently crushed to break up clods, passed through a 1 mm sieve, and stored for analysis. Total nitrogen, total carbon, and total organic carbon (TOC) were determined by weighing 0.3 g of soil and analyzing it with a Soli TOC cube analyzer (Elementar, Langenselbold, Germany). Soil organic matter content was calculated as TOC × 1.724, following the Van Bemmelen factor assumption that organic matter contains 58% carbon (Nelson & Sommers 1996). Available phosphorus, exchangeable potassium, exchangeable calcium, and exchangeable magnesium were determined by weighing 2.0 g of soil into a 50 mL conical flask, adding 20 mL of Mehlich No. 3 extractant, and shaking at 200 rpm for 5 min. The suspension was filtered, and the filtrate analyzed using an inductively coupled plasma–optical emission spectrometer (iCAP 7000 Series, Thermo Fisher Scientific, Waltham, MA, USA).

Statistical analysis

To appropriately model the hierarchical structure of the data and account for site-level heterogeneity, we employed linear mixed-effects models (LMMs), constructed using the lme4 package (version 1.1.37) in R (version 4.3.2, R Core Team, Austria). This approach was necessary due to the nested experimental design, where orchard identity was nested within cultivation method (organic vs. conventional). LMMs enable the partitioning of variance into fixed and random components, providing unbiased estimates of treatment effects while adjusting for the non-independence of observations collected within the same orchard. Cultivation method was treated as a fixed effect, and orchard identity (nested within cultivation method) was included as a random effect. Separate models were constructed for each fruit quality trait: antioxidant capacity, total phenolic content, total flavonoid content, and vitamin C content.

To assess fixed effects (cultivation method), we computed estimated marginal means (EMMs) for each cultivation method using the emmeans package (R package version 1.6.1), which adjusts for the structure of the random effects. The statistical significance of fixed effects was evaluated using Type II Wald χ^2 tests, implemented in the car package. The contribution of the random effect (orchard) was tested using likelihood ratio tests by comparing full and reduced models (excluding the random term) via the anova function.

To evaluate model explanatory power, marginal R^2 (R^2m) and conditional R^2 (R^2c) were calculated using the performance package, following the method of Nakagawa & Schielzeth (2013). R^2m quantifies variance explained by fixed effects alone, while R^2c accounts for both fixed and random effects.

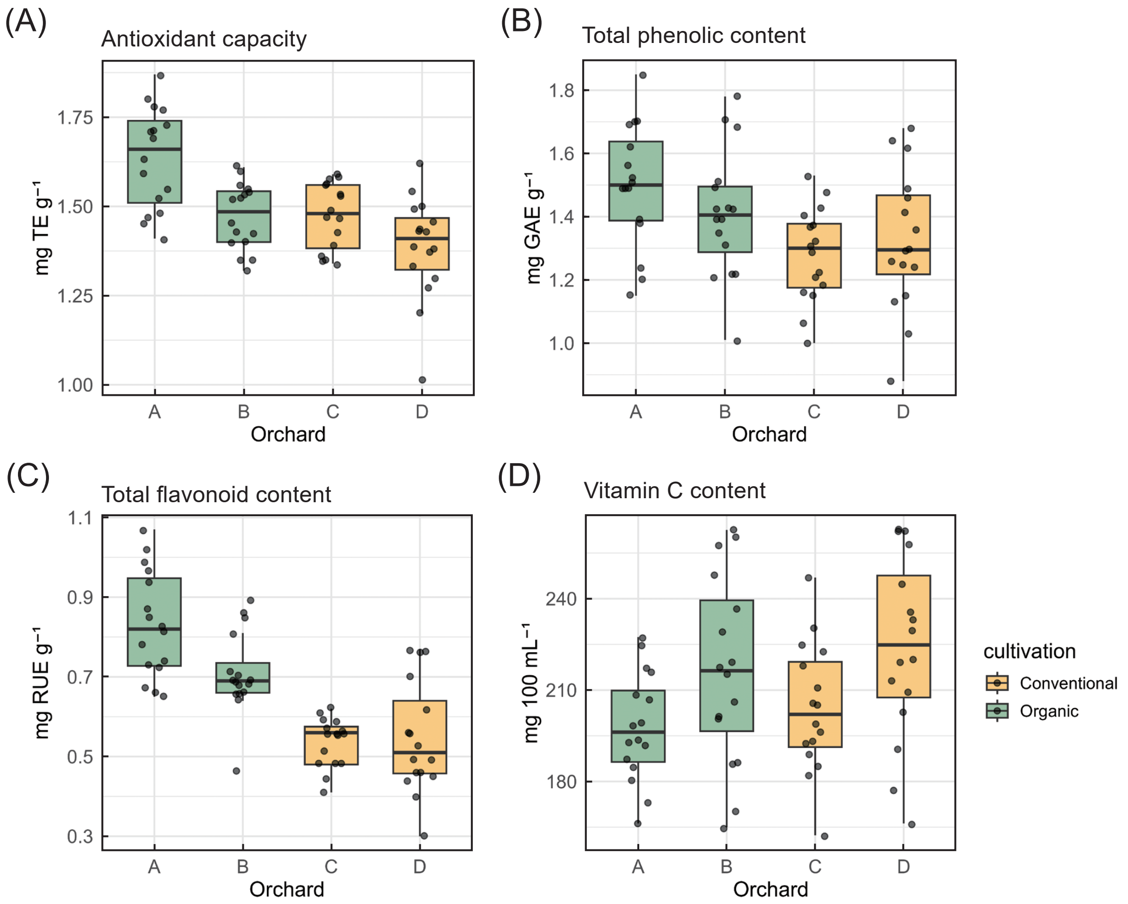
To visualize orchard-level variation within cultivation methods, boxplots were generated using the ggplot2 package. Finally, Pearson's correlation coefficients were calculated using the base R cor function to assess associations between antioxidant capacity and the three phytochemical traits. Statistical significance was determined at $P < 0.05$.

RESULTS

Descriptive overview of functional quality indicators

A total of 64 Indian jujube fruit samples were collected from four orchards, each representing one of two cultivation methods- conventional (orchards C and D) or organic (orchards A and B). Each orchard contributed 16 fruit samples. Four functional indicators were evaluated: antioxidant capacity, total phenolic content, total flavonoid content, and vitamin C content.

Descriptive statistics for all variables across the four orchards are summarized in Fig. 2. In terms of antioxidant capacity, Orchard A (organic) exhibited the highest mean value (1.64 ± 0.14 mg TE g^{-1}), followed by Orchard C



(E)

Cultivation method	Orchard	Antioxidant capacity (mg TE g^{-1})	Total phenolic content (mg GAE g^{-1})	Total flavonoid content (mg RUE g^{-1})	Vitamin C content (mg 100 mL^{-1})
Organic	A	1.64 ± 0.14	1.50 ± 0.19	0.83 ± 0.13	197.9 ± 17.8
Organic	B	1.47 ± 0.09	1.41 ± 0.20	0.71 ± 0.11	216.3 ± 31.1
Conventional	C	1.47 ± 0.09	1.28 ± 0.15	0.54 ± 0.06	203.8 ± 21.2
Conventional	D	1.38 ± 0.14	1.32 ± 0.22	0.55 ± 0.14	224.1 ± 30.2

Fig. 2. (A–D) Boxplots of antioxidant capacity, total phenolic content, total flavonoid content, and vitamin C content in Indian jujube fruits from four orchards (n = 16 per orchard). (E) Descriptive statistics (mean ± SD) for each functional component under different cultivation methods and orchards. Orchards A and B were organic; orchards C and D were conventional. Boxplots show the interquartile range (IQR, box), median (horizontal line), whiskers (1.5 × IQR), and individual samples (jittered points). Units: antioxidant capacity (mg TE g^{-1}), total phenolic content (mg GAE g^{-1}), total flavonoid content (mg RUE g^{-1}), vitamin C (mg 100 mL^{-1}).

(1.47 ± 0.09 mg TE g^{-1}) and Orchard B (1.47 ± 0.09 mg TE g^{-1}), with Orchard D (conventional) showing the lowest value (1.38 ± 0.14 mg TE g^{-1}).

For phenolic content, Orchard A again showed the highest mean value (1.50 ± 0.19 mg GAE g^{-1}), followed by Orchard B (1.41 ± 0.20

mg GAE g^{-1}), Orchard D (1.32 ± 0.22 mg GAE g^{-1}), and Orchard C (1.28 ± 0.15 mg GAE g^{-1}). Total flavonoid content showed a more pronounced difference between cultivation types, with organic orchards A and B reporting higher mean values (0.83 ± 0.13 and 0.71 ± 0.10 mg RUE g^{-1}, respectively) compared to conventional orchards C and D (0.54 ± 0.06 and 0.55 ± 0.14 mg RUE g^{-1}, respectively) (Fig. 2).

The vitamin C content varied across orchards without a clear pattern associated with cultivation method. Orchard D exhibited the highest mean value (224.1 ± 30.2 mg 100 mL^{-1}), followed by Orchard B (216.3 ± 31.1 mg 100 mL^{-1}), Orchard C (203.8 ± 21.2 mg 100 mL^{-1}), and Orchard A (197.9 ± 17.8 mg 100 mL^{-1}) (Fig. 2). These results suggest that, while some functional components, such as flavonoids, appear more responsive to cultivation method, orchard-specific factors may influence like vitamin C and others.

Effect of cultivation method on functional quality of indian jujube fruits

The influence of cultivation method on the functional quality of Indian jujube fruits was assessed using linear mixed-effects models, with orchard nested within cultivation method as a random effect. As shown in Table 1, the fixed effect of cultivation method was not statistically significant for antioxidant capacity (P = 0.313), total phenolic content (P = 0.225), or vitamin C content (P = 0.628). However, the difference in total flavonoid content between organic and conventional samples (P = 0.053) approached statistical significance (P = 0.05).

Estimated marginal means were also illustrated in Table 1. Although without significance, organic fruits showed higher mean values than conventional fruits for antioxidant capacity (1.55 vs. 1.43 mg TE g^{-1}), total phenolic content (1.45 vs. 1.30 mg GAE g^{-1}), and total flavonoid content (0.77 vs. 0.54 mg RUE g^{-1}). In contrast, the mean vitamin C content of organic fruits was slightly lower than that of conventional fruits (207.1 vs. 213.9 mg 100 mL^{-1}), and this difference was also not statistically significant.

The marginal R^2 values from Nakagawa's method ranged from 0.077 to 0.267 across models, suggesting that cultivation method alone explained a modest proportion of the total variance. Conditional R^2 values were higher (0.338 to 0.443), indicating that orchard-level random effects also contributed meaningfully to the observed variation (Table 1).

Variation at the orchard level in phytochemical profiles

The random effect using the linear mixed-effects models is shown in Table 2. The variance components for orchard-level effects were found to be statistically significant for antioxidant capacity (P < 0.001), total flavonoid content (P = 0.013), and vitamin C content (P = 0.015). Conversely, total phenolic content did not exhibit a significant random effect at the orchard level (P = 0.347).

Table 1. Estimated marginal means and statistical significance of functional compounds in Indian jujube fruits under different cultivation methods.

Response variable	Organic (EMM ± *SE*)[z]	Conventional (EMM ± *SE*)	*P*-value	Marginal R^{2y}	Conditional R^{2x}
Antioxidant capacity (μmol TE g^{-1})	1.55 ± 0.08	1.43 ± 0.08	0.313	0.149	0.443
Total phenolic content (mg GAE g^{-1})	1.45 ± 0.10	1.30 ± 0.10	0.225	0.267	0.338
Total flavonoid content (mg RUE g^{-1})	0.77 ± 0.06	0.54 ± 0.06	0.053	0.182	0.360
Vitamin C content (mg 100 mL^{-1})	207.1 ± 9.6	213.9 ± 9.6	0.628	0.077	0.410

[z] Estimated Marginal Means (EMM) ± Standard Error (*SE*) derived from linear mixed-effects models.
[y] Marginal R^2 represents the proportion of variance explained by the fixed effects (cultivation method) alone.
[x] Conditional R^2 represents the proportion of variance explained by both fixed effects and random effects (orchard). R^2 values were calculated using Nakagawa's method.

Soil physicochemical properties of the orchards

Soil physicochemical properties of the four orchards are summarized in Table 3. Soil pH values were uniformly alkaline, ranging from 7.91 to 7.99. Organic matter content was highest in Orchard B (2.961%), followed closely by Orchard A (2.901%), and was lower in Orchard C (2.137%) and lowest in Orchard D (1.995%). Total carbon content was also highest in Orchard A (1.874%), followed by Orchard B (1.864%), Orchard C (1.390%), and Orchard D (1.255%). Total nitrogen content was highest in Orchard A (0.255%), followed by Orchard B (0.173%), Orchard D (0.132%), and Orchard C (0.130%). Available phosphorus varied markedly, with Orchard C (923.9 mg kg^{-1}) and Orchard A (618.8 mg kg^{-1}) showing much higher concentrations than Orchard B (51.6 mg kg^{-1}) and Orchard D (39.1 mg kg^{-1}). Exchangeable potassium was highest in Orchard C (643.6 mg kg^{-1}) and lowest in Orchard D (120.1 mg kg^{-1}). Exchangeable calcium ranged from 2,424.8 mg kg^{-1} in Orchard D to 3,703.1 mg kg^{-1} in Orchard A, whereas exchangeable magnesium was highest in Orchard C (1,030.8 mg kg^{-1}) and lowest in Orchard B (572.4 mg kg^{-1}).

Correlations among functional compounds

Pearson correlation analyses were performed to investigate relationships among antioxidant capacity, total phenolic content, total flavonoid content, and vitamin C content across all samples (n = 64). As shown in Table 4, antioxidant capacity was strongly and positively correlated with total phenolic content (r = 0.85, P < 0.001) and total flavonoid content (r = 0.79, P < 0.001). Total phenolic and flavonoid contents were also significantly correlated (r = 0.76, P < 0.001). In contrast, vitamin C content showed no significant correlation with antioxidant capacity (r = 0.12, P = 0.35), total phenolic content (r = 0.08, P = 0.48), or flavonoid content (r = 0.10, P = 0.42). These results indicate that phenolic and flavonoid compounds likely contribute substantially to the antioxidant capacity of Indian jujube fruits. In contrast, vitamin C may play a less prom-

Table 2. Random effects of orchard nested within cultivation method on antioxidant capacity, total phenolic content, total flavonoid content, and vitamin C content in Indian jujube fruits (n = 64).

Response variable	Random effect variance (σ^2)[z]	P-value[y]
Antioxidant capacity	0.0077	< 0.001
Total phenolic content	0.0004	0.347
Total flavonoid content	0.0021	0.013
Vitamin C content	25.43	0.015

[z] Random effects are estimated using the restricted maximum likelihood method in linear mixed-effects models.
[y] P-values are based on likelihood ratio tests comparing models with and without orchard-level random intercept.

Table 3. Soil physicochemical properties in four orchards.

Orchard	pH (1 : 1 soil : water)	Organic matter (%)	Total carbon (%)	Total nitrogen (%)	Available phosphorus (mg kg^{-1})	Exchangeable potassium (mg kg^{-1})	Exchange-able calcium (mg kg^{-1})	Exchangeable magnesium (mg kg^{-1})
A	7.97	2.901	1.874	0.255	618.8	340.9	3,703.1	966.9
B	7.91	2.961	1.864	0.173	51.6	158.5	3,378.8	572.4
C	7.91	2.137	1.390	0.130	923.9	643.6	3,611.3	1,030.8
D	7.99	1.995	1.255	0.132	39.1	120.1	2,424.8	702.2

Table 4. Pearson correlation coefficients among antioxidant capacity, total phenolic content, total flavonoid content, and vitamin C content in Indian jujube fruits (n = 64).

Response variables	Antioxidant capacity	Total phenolic content	Total flavonoid content	Vitamin C content
Antioxidant capacity	1			
Total phenolic content	0.85 ($P < 0.001$)[z]	1		
Total flavonoid content	0.79 ($P < 0.001$)	0.76 ($P < 0.001$)	1	
Vitamin C content	0.12 ($P = 0.35$)[y]	0.08 ($P = 0.48$)	0.10 ($P = 0.42$)	1

[z] Correlation significant at $P < 0.001$.
[y] Correlation not statistically significant ($P > 0.05$).

inent role in determining antioxidant activity under the tested conditions.

DISCUSSION

Influence of cultivation method on functional quality of indian jujube fruits

In this study, cultivation method (organic vs. conventional) did not exert a statistically significant effect on any of the measured quality traits, including antioxidant capacity, total phenolic content, total flavonoid content, and vitamin C content (Table 1). While fruits from organic orchards tended to exhibit slightly higher mean levels, for example, antioxidant capacity and flavonoid content, these differences were modest. Only total flavonoid content approached statistical significance ($P = 0.053$), a value close to the conventional threshold ($P = 0.05$) (Table 1). The tendency for flavonoid content is likely linked to typical organic practices, where reduced synthetic inputs and increased exposure to environmental stressors stimulate secondary metabolite synthesis (Jan *et al*. 2021; Khan *et al*. 2025). Specifically, practices such as limited pesticide use and lower nitrogen availability can heighten plant defense responses, leading to the upregulation of phenylpropanoid pathways responsible for polyphenol biosynthesis and the accumulation of enhanced antioxidant compounds as part of the plant's adaptive strategy (Stewart *et al*. 2001; Krey *et al*. 2020). Nevertheless, under the specific conditions examined in this study, cultivation system alone did not appear to be a dominant factor shaping fruit phytochemical profiles.

Orchard-Level variation in phytochemical composition and its implications

The linear mixed-effects models effectively captured the heterogeneity at the orchard level. The random effects of orchard nested within cultivation method accounted for a substantial proportion of the total variance, particularly for antioxidant capacity ($P < 0.001$), total flavonoid content ($P = 0.013$), and vitamin C content ($P = 0.015$) (Table 2). In this study, orchard-level variation is defined as the cumulative influence of site-specific environmental conditions and managerial practices that are not explicitly represented by the binary classification of cultivation methods (organic vs. conventional). These factors include soil physicochemical properties, orchard history, microclimatic differences, and other unmeasured field-specific variables. Such factors can modulate plant physiological processes and influence the accumulation of phytochemicals (Stracke *et al*. 2009; Rocchetti *et al*. 2022). In essence, orchard-level variation reflects the integrated field context in which a crop is grown, and its effects can even surpass those attributable to cultivation system categories.

Among the potential contributors to orchard-level variation identified above, measured soil nutrient profiles (Table 3) may offer plausible explanatory cues. For example, the higher antioxidant capacity and total flavonoid content observed in organic Orchard A relative to Orchard B (Fig. 2) may be attributable to the combined effects of several edaphic factors. While the two orchards exhibited similar organic matter and total carbon levels, Orchard A had

higher concentrations of nitrogen, phosphorus, potassium, and magnesium (Table 3). From a physiological perspective, a balanced supply of macronutrients and magnesium enhances chlorophyll biosynthesis and photosynthetic efficiency, thereby increasing the flux of fixed carbon toward sink organs (Farhat *et al*. 2016; Wang *et al*. 2024). This expanded carbon pool supports the energetically demanding pathways for flavonoid biosynthesis (Deng *et al*. 2019), potentially explaining the superior flavonoid content in Orchard A. Such relationships between nutrient and secondary metabolism linkages have been consistently reported in fruit crops (Pott *et al*. 2019), reinforcing nutrient balance as one of the reliable drivers of orchard-level differences.

In contrast, the conventional group highlights a different facet of orchard-level variation. Orchard C displayed higher soil organic matter and multiple macronutrients than Orchard D (Table 3). Yet, the two orchards showed no appreciable difference in phytochemical metrics (Fig. 2). This finding aligns with the hypothesis that excessive nutrient supply may not translate into proportional quality gains in conventional systems (Kim *et al*. 2020; Milošević *et al*. 2022). Possible explanations include the lack of a robust soil microbial network to facilitate nutrient transformation and uptake, which is often more developed in long-term organic systems (Rodríguez-Ortiz *et al*. 2022; Wang *et al*. 2022). This observation highlights that nutrient abundance alone may not be sufficient. The bioavailability of nutrients and the efficiency of their physiological integration are equally critical.

Although we lack direct measurements of canopy light or temperature profiles, numerous studies in perennial fruit crops suggest that site-specific microclimate and light likely interact with soil nutrient status to modulate phytochemical outcomes (Vaneková *et al*. 2020; Han *et al*. 2023). Future research should integrate more environmental monitoring, such as canopy light mapping, leaf water potential tracking, and soil microbial community profiling, to better understand how microclimate-nutrient interactions drive phytochemical variation across orchards. Taken together, our results reinforce the conclusion that orchard-level variation can exert a stronger influence on the functional quality of Indian jujube than cultivation method alone.

Relationships between functional compounds and antioxidant capacity

Antioxidant capacity was strongly and positively correlated with both total phenolic content (r = 0.81, P < 0.001) and total flavonoid content (r = 0.78, P < 0.001), indicating that these two classes of polyphenolic compounds are the principal contributors to the antioxidant potential of Indian jujube fruits. This finding is consistent with the established role of phenolics and flavonoids as major radical scavengers in plant-derived foods (Mustafa *et al*. 2010; Muflihah *et al*. 2021). Previous studies on *Ziziphus* species have likewise found phenolic content to be the dominant determinant of antioxidant activity. For instance, Zhang *et al*. (2010) reported a strong association between phenolic concentration and antioxidant potential in Chinese jujube extracts.

In contrast, vitamin C content showed no significant correlation with antioxidant capacity (r = -0.06, P = 0.61), nor with total phenolic content (r = 0.08, P = 0.48) or flavonoid content (r = 0.10, P = 0.42). This suggests that vitamin C plays a comparatively minor role in the antioxidant capacity of Indian jujube under the conditions studied. Although vitamin C is recognized as an important antioxidant, its contribution can vary significantly across species, cultivars, and environmental contexts (Zhang *et al*. 2010; Wojdyło *et al*. 2016).

From an application perspective, these findings suggest that enhancing the functional quality of Indian jujube may be more effectively achieved by promoting polyphenol biosynthesis through targeted stress induction or optimized agronomic practices, rather than by focusing solely on increasing vitamin C levels. Breeding efforts or orchard management strategies that

select for or enhance phenolic and flavonoid accumulation may therefore offer greater nutritional benefits and functional consistency.

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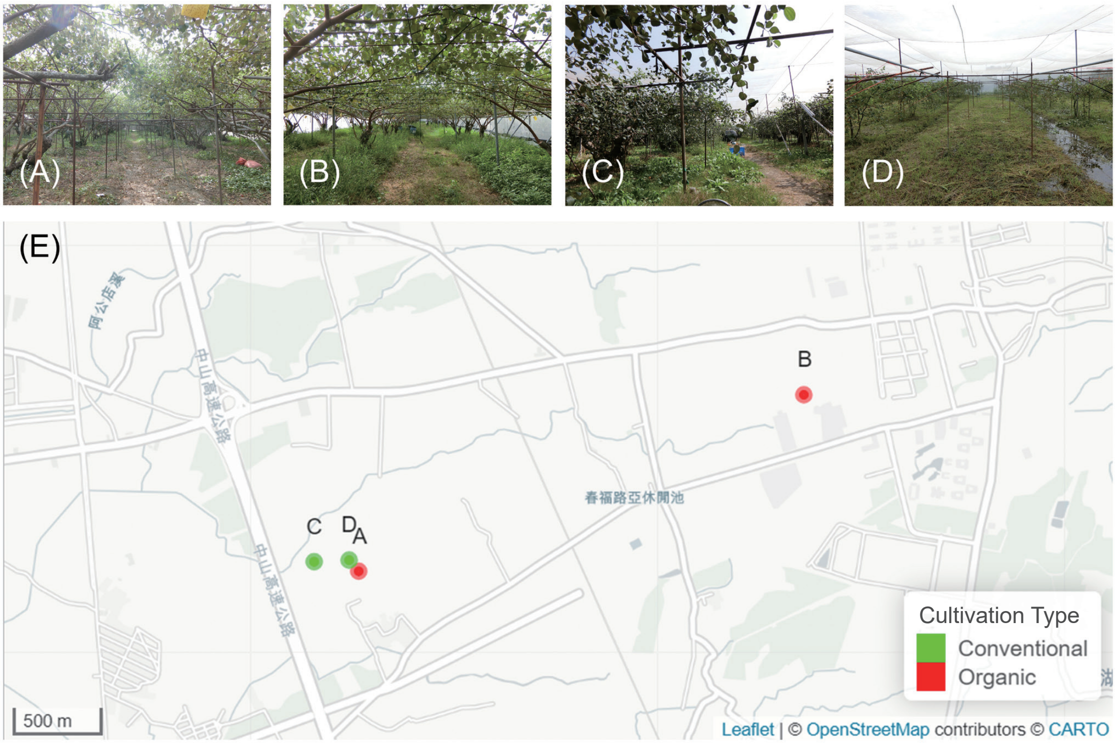
Appendix 1. Geographic location and cadastral information of the four orchards used in this study, including cultivation type, area, cadastral parcel numbers, and global positioning system (GPS) coordinates.

Orchard	Cultivation type	Area (hectare)	Location (cadastral parcels)	GPS
A	Organic	0.4	Guanshui 293, 293-1, 293-2	22.7802,120.3281
B	Organic	0.25	Antong 0705-0000	22.7890,120.3521
C	Conventional	0.4	Guanshui 300, 300-1, 300-2	22.7807,120.3257
D	Conventional	0.3	Guanshui 0283	22.7808,120.3276



Appendix 2. (A–D) Photographs of the four Indian jujube orchards at the Kaohsiung Yanchao Long-Term Ecological Research Station. Orchards A and B were managed organically, while C and D were under conventional cultivation. (E) Location map of the orchards, generated with the R package leaflet.

果園層級變異與栽培方式對印度棗 (*Ziziphus mauritiana*) 機能品質之影響

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摘要

洪子桓、張家華、陳秋樺、陳素滿、徐敏記、郭展宏。2025。果園層級變異與栽培方式對印度棗 (*Ziziphus mauritiana*) 機能品質之影響。台灣農業研究 74(4):525–539。

本研究探討栽培方式 (有機與慣行) 與果園層級變異對印度棗 (*Ziziphus mauritiana*) 果實機能品質的影響。研究從臺灣 4 個果園 (2 個有機果園與 2 個傳統果園) 中採集共 64 個果實樣本，分析其抗氧化力、總酚含量、總黃酮含量及維生素 C 含量。雖然有機果實在抗氧化力、酚類及黃酮類化合物含量上的平均值略高，但線性混合效應模型顯示栽培方式對各項機能指標無顯著統計影響。相較之下，果園層級的隨機效應解釋了總變異中的重要比例，尤其是在抗氧化力、總黃酮及維生素 C 方面。同一栽培方式下的不同果園間，亦觀察到顯著差異，顯示局部環境或管理條件可能比栽培系統本身對果實化學特徵方面具有更大的影響。果園間存在明顯的土壤養分差異，為觀察到的植化素變異提供了合理解釋線索。皮爾森相關性分析顯示抗氧化力與總酚及總黃酮含量呈顯著正相關，而維生素 C 則與其他指標無顯著相關，推測酚類與黃酮類化合物可能是印度棗抗氧化潛力的主要貢獻者。

關鍵詞：印度棗、機能品質、栽培方式、果園層級變異、線性混合效應模型。

投稿日期：2025 年 6 月 30 日；接受日期：2025 年 9 月 12 日。
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台灣農業研究 (J. Taiwan Agric. Res.) 74(4):541–556 (2025)
https://doi.org/10.6156/JTAR.202512_74(4).0011

Research Article

Utilization of an Intelligent Electrohydrodynamic Dryer to Improve Drying Efficiency of Camellia Seeds

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Abstract

Tseng, H. E., Y. L. Lee, Y. H. Lee, K. C. Lin, and W. J. Su. 2025. Utilization of an intelligent electrohydrodynamic dryer to improve drying efficiency of camellia seeds. J. Taiwan Agric. Res. 74(4):541–556.

This study investigated the application of electrohydrodynamic (EHD) drying technology, utilizing a self-developed intelligent EHD dryer for drying the seeds of *Camellia brevistyla*, a native species of Taiwan, to compare its performance with the traditional sun drying method. The effects of the EHD dryer on drying efficiency of the seeds, and the resulting cold-pressed oil quality, including acid value (AV) and peroxide value (POV), and oil oxidative stability index (OSI) as well, were evaluated. Two batches of camellia seeds were conducted, and the EHD drying was executed at 42℃ to reach the average high temperature during the sun-drying period. Results showed that the intelligent EHD method significantly enhanced drying efficiency, resulting in a shorter drying time (48 h) compared to the sun drying method (9 d). The intelligent EHD dryer exhibited uniform drying and minimized the need for manual seed turning. Oil quality analyses revealed that the intelligent EHD drying exhibited significantly lower AV and POV values compared to the oil obtained via traditional sun drying, indicating superior oil quality. The OSI and sensory evaluation of flavor characteristics were similar for both methods. In summary, applying the intelligent EHD dryer to dry camellia seeds improves drying efficiency and enhances the quality of the pressed oil, thereby demonstrating high potential for further utilization.

Key words: *Camellia brevistyla*, Camellia seeds, Seeds drying method, Electrohydrodynamic drying, Oil quality.

INTRODUCTION

The oil-seed camellia tree (*Camellia oleifera* and *C. brevistyla*), a member of the Theaceae family, is an evergreen shrub or small tree cultivated in Taiwan for its high oil-yielding seeds, commonly referred to as camellia seeds. These seeds contain 30–50% oil, which is extracted to produce what is known locally as “bitter tea oil,” and internationally as camellia oil or Tsubaki oil. For consistency, the term “camellia oil” is used throughout this paper. In Taiwan, *C. oleifera* is primarily grown in central, southern, and eastern mountain regions due to its tolerance of higher temperatures, while *C. brevistyla* is better adapted to cooler climates and is mainly cultivated in northern mountainous areas such as Miaoli. These species exhibit distinct physiological traits- for example, *C. brevistyla* is commonly used as a rootstock for

Received: May 27, 2025; Accepted: September 12, 2025.
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grafting *C. oleifera*, whereas the reverse combination has limited success.

It is vital to distinguish camellia oil from tea seed oil derived from *C. sinensis*, the tea plant primarily cultivated for beverages. Tea seed oil has a lower oil content (~ 20%) and a fatty acid composition closer to that of sesame or peanut oil (Hsieh *et al*. 2013). In contrast, camellia oil resembles olive oil in its high oleic acid content (70–80%) (Haiyan *et al*. 2007), which is associated with cardiovascular health benefits (Kris-Etherton 1999; Sales-Campos *et al*. 2013). In addition, the polyphenolic compounds in camellia oil contribute antioxidant activity and enhance oxidative stability (Cicerale *et al*. 2010; Ajal *et al*. 2021), making it a nutritionally and functionally valuable oil.

Due to Taiwan's hot and humid climate, freshly harvested camellia seeds are highly susceptible to infestation by pests, mold, and bacteria, all of which can degrade seed quality and negatively impact the resulting oil. Drying is therefore an essential postharvest step to reduce seed moisture, inhibit microbial activity, and ensure oil stability during storage. Sun drying is the most commonly used method due to its simplicity and zero energy cost. However, it has several well-documented drawbacks, including lengthy drying times, high labor demands, poor process control, and large spatial requirements. Additionally, exposure to dust, insects, and other contaminants during open-air drying can compromise oil quality (Kooli *et al*. 2007; Zhang *et al*. 2016). A more efficient, hygienic, and controlled drying method is thus necessary to improve oil retention and maintain the quality of camellia seeds. Beyond preservation, an optimized drying process also contributes to improved appearance, reduced shrinkage, higher rehydration capacity, and better packaging and transport efficiency (Kamarulzaman *et al*. 2021).

To address the limitations of traditional drying methods, electrical and electrochemical technologies have been increasingly explored in the food processing industry. Among them, pulsed electric fields (PEF), moderate electric fields (MEF), and high-voltage electric fields (HVEF) have shown potential in applications such as microbial inactivation, enzyme modulation, and structural modification of food matrices. In particular, HVEF has been applied to drying processes, where static electric fields or high-voltage discharges accelerate moisture migration and reduce microbial activity at relatively low temperatures. These non-thermal drying approaches have been shown to improve drying efficiency and product quality while minimizing thermal damage, as demonstrated in the postharvest treatment of mushrooms and other fresh produce (Lin *et al*. 2020). In that study, high-voltage electric field (HVEF) treatment was applied during storage of *Pleurotus eryngii*, effectively delaying browning, reducing microbial growth, and extending shelf life from 9 to 21 d. The application of an electric field not only maintained the firmness and color of mushrooms but also significantly reduced storage losses, highlighting the practical benefits of non-thermal electrical preservation technologies in highly perishable agricultural products.

Electrohydrodynamic (EHD) drying, derived from the principles of high-voltage electrical discharge, represents a more recent and innovative advancement in non-thermal drying technology. Unlike conventional HVEF systems, EHD drying utilizes corona discharge to generate ion wind- a stream of charged particles that enhances surface moisture removal under ambient conditions. While this mechanism has been studied in experimental contexts, its application in practical-scale drying systems remains limited. The present study, therefore, contributes original work by integrating an intelligent EHD drying system into agricultural drying applications, specifically for camellia seeds. As illustrated in Fig. 1A, the EHD drying mechanism is based on corona discharge between a high-voltage emitter and a grounded collector, which creates a unidirectional ion wind that facilitates convective mass transfer. In this study, a wire-type emitter electrode was adopted (Fig. 1B), allowing for stable discharge and uniform airflow across the

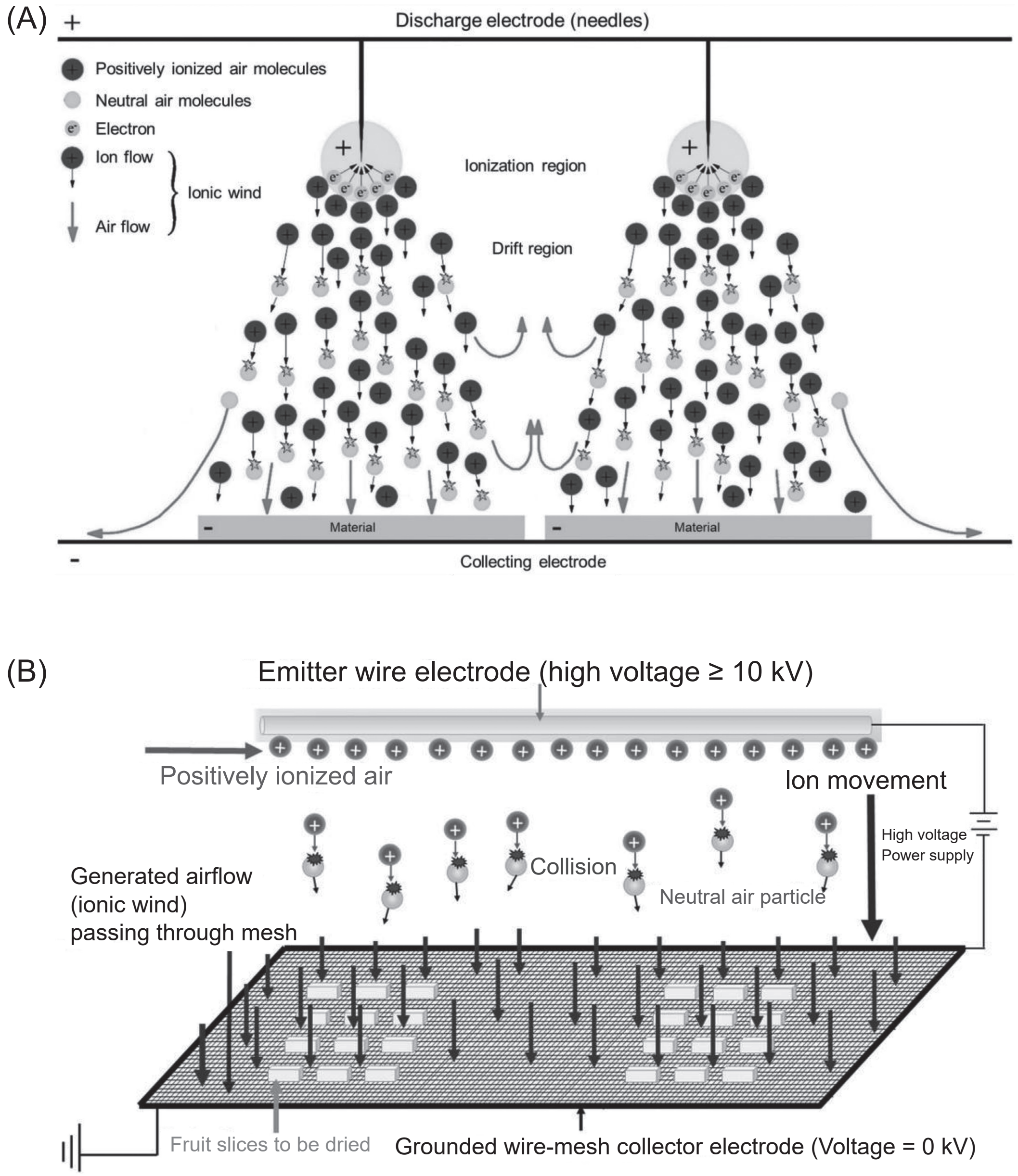


Fig. 1. Schematic illustration of the electrohydrodynamic (EHD) drying principle and system configuration. (A) Principle of ion wind drying: When a high voltage is applied between the emitter and the grounded collector in a gaseous medium, corona discharge occurs near the emitter, resulting in ionization of air molecules. The movement of charged ions toward the collector generates a unidirectional secondary airflow, known as ion wind, which enhances moisture removal from the surface of the material. (B) EHD drying method: The EHD system used in this study consists of a wire-type emitter electrode connected to a direct current (DC) high-voltage power supply and a grounded mesh collector. The material to be dried is placed on the collector surface, where it is directly exposed to the ion wind, facilitating uniform, energy-efficient drying at ambient temperature.

drying surface and enabling consistent moisture removal while preserving product quality.

MATERIALS AND METHODS

Camellia fruit materials and experimental design

To evaluate the performance of the EHD drying system, this study utilized Taiwan-native oil-seed camellia (*C. brevistyla*) fruits as the experimental material. Fresh fruits were harvested from Sanwan Township, Miaoli County, at the end of 2023. Two batches of fruits were collected on October 26 and November 3, respectively. The fresh fruits were stored in a refrigerator at 4℃ prior to testing. The initial moisture content of the fresh oil-seed camellia fruits was approximately 53–55%. Two drying methods were evaluated: the traditional sun drying method (used as the control) and the EHD drying method. The drying temperature for the EHD system was set to 42℃, based on the surface average high temperatures achievable of camellia seeds with the sun-drying method (empirical experience), also the environmental temperature typically used for drying peanuts.

Design and structure of the intelligent EHD dryer

The intelligent EHD dryer developed in this study was designed for agricultural drying applications and evaluated using camellia seeds as a model material. The system comprises two key components: (1) the main drying structure and (2) the intelligent control system.

Main drying structure

The physical components of the EHD dryer include a drying chamber, a hot-air heating unit, an adjustable airflow channel system, an EHD drying field generator, and sensor integration modules. A three-dimensional schematic of the dryer, along with part names and component numbers, is shown in Fig. 2A and detailed in Table 1. System specifications are listed in Table 2.

To improve drying uniformity, the chamber was equipped with a multi-mode airflow system that supports four drying modes: top-down, bottom-up, internal circulation, and static resting (Fig. 2B). This airflow variability allows hot air to be adjusted according to the stacking patterns and material density of the seeds, preventing localized overheating and uneven drying. The system also includes a tempering function- intermittent operation during the drying cycle- to gradually penetrate heat into the material and prevent hull cracking due to overdrying.

The EHD drying field is operated under an applied electric field of 10–40 kV, generating an ion wind that accelerates surface moisture evaporation while enhancing convective heat transfer. This non-thermal mechanism enables rapid moisture removal at lower temperatures, reducing the risk of oil oxidation and preserving the nutritional quality of camellia seeds.

Intelligent control system

The dryer integrates a human-machine interface (HMI) with a programmable logic controller (PLC) to support real-time monitoring and adjustment of key parameters such as temperature, humidity, and airflow (Fig. 3A). Control point distribution for temperature and humidity regulation within the chamber is illustrated in Fig. 3B (Chinese-language interface). Multiple temperature and humidity sensors are embedded at various positions inside the chamber (Fig. 3C) to ensure uniform hot-air distribution. A weight sensor tracks the real-time mass loss of seeds to estimate moisture reduction. At the same time, environmental monitoring modules measure both indoor and outdoor conditions to dynamically optimize drying settings. The system also features one-touch start, automated airflow switching, and heater modulation, allowing the dryer to adapt in real-time to different ambient conditions. Remote monitoring and data logging functions are included to facilitate performance evaluation and operational traceability.

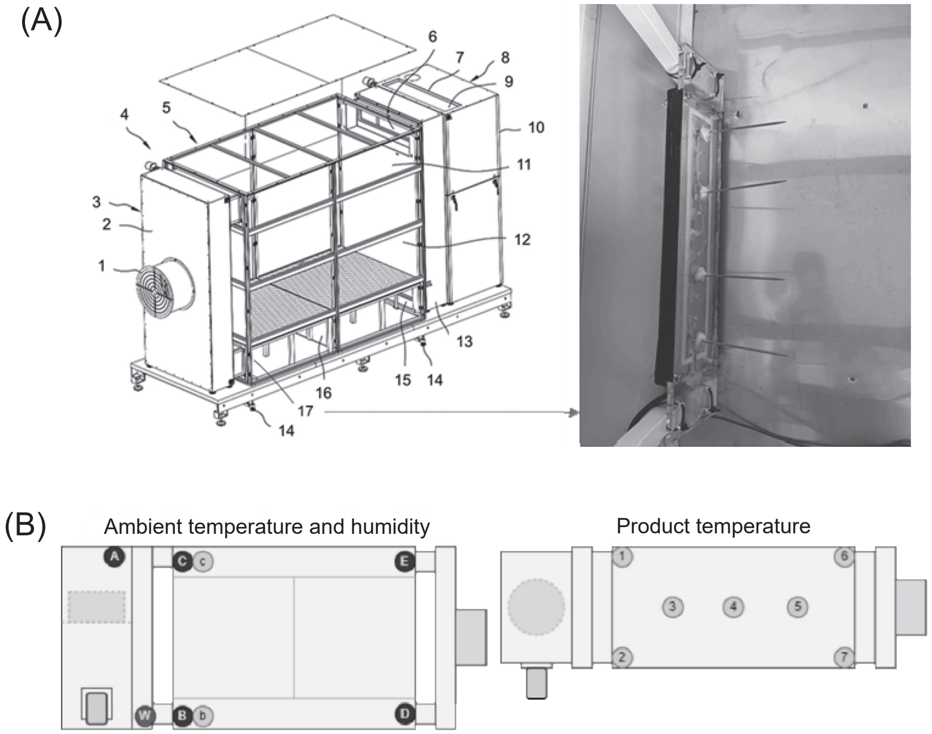


Fig. 2. Structural design and drying modes of the intelligent electrohydrodynamic (EHD) dryer. (A) Three-dimensional schematic of the intelligent EHD dryer, showing the main structural components. The names of components are shown in Table 1. (B) Illustration of four adjustable airflow modes: top-down, bottom-up, internal circulation, and static resting. These airflow configurations enable flexible hot-air routing to enhance drying uniformity based on material distribution. Temperature and humidity sensors are installed at A, B, C, D, E, and W. In addition, temperature sensors are installed at b and c. Numbers 1 to 7 represent the location of the temperature sensor.

Table 1. Component names and part numbers of the intelligent electrohydrodynamic (EHD) dryer.

No.	Component	No.	Component
1	Negative-pressure fan	9	Air inlet valve
2	Second air duct	10	Positive-pressure fan
3	Exhaust system	11	Upper airflow chamber
4	Damper actuator	12	Drying material chamber
5	Drying chamber body	13	First air duct
6	Upper air outlet	14	Weight sensor
7	Air inlet	15	Lower air outlet
8	Heating cabinet (with pressure unit)	16	Lower airflow chamber
		17	Positively ionized air molecules

Sun drying procedure of camellia seeds

The sun drying procedure was designed to simulate traditional practices commonly used by local farmers. Each day, drying was conducted from 8:00 to 17:00. During the first two days of drying, the pericarp of the fresh camellia fruits naturally cracked and was manually removed as needed to extract the seeds, which were then directly exposed to sunlight. From the third day onward, the sun-dried camellia seeds were

Table 2. Specifications of the intelligent intelligent electrohydrodynamic (EHD) dryer.

Item	Specification
Overall dimensions	435.0 cm × 130.5 cm × 215.5 cm (height excluding exhaust pipe and floor scale)
Drying chamber size	245.5 cm × 130.5 cm × 215.5 cm
Maximum stacking depth	Approximately 120 cm
Heating system	Diesel burner with heat exchanger
Fan specifications	Main fan: 1.5 hp; Negative-pressure fan (powered by energy storage system): 1.0 hp (both equipped with inverters)
Drying modes	1. Bottom-blowing/top-suction mode 2. Top-blowing/bottom-suction mode 3. Internal circulation mode 4. Static (resting) mode
Control modes	1. Human-machine interface with smartphone app for remote control 2. Manual/automatic control modes
Material weighing	8-ton floor scale (Load Cell: 2 tons × 4 units)
Sensors	1. Six temperature-humidity sensors 2. Nine temperature sensors (including seven placed within the drying material)
EHD electrodes configuration	Operating at 9 kV, 350 μA; positioned above and below drying layers to enhance ionic wind-assisted convection

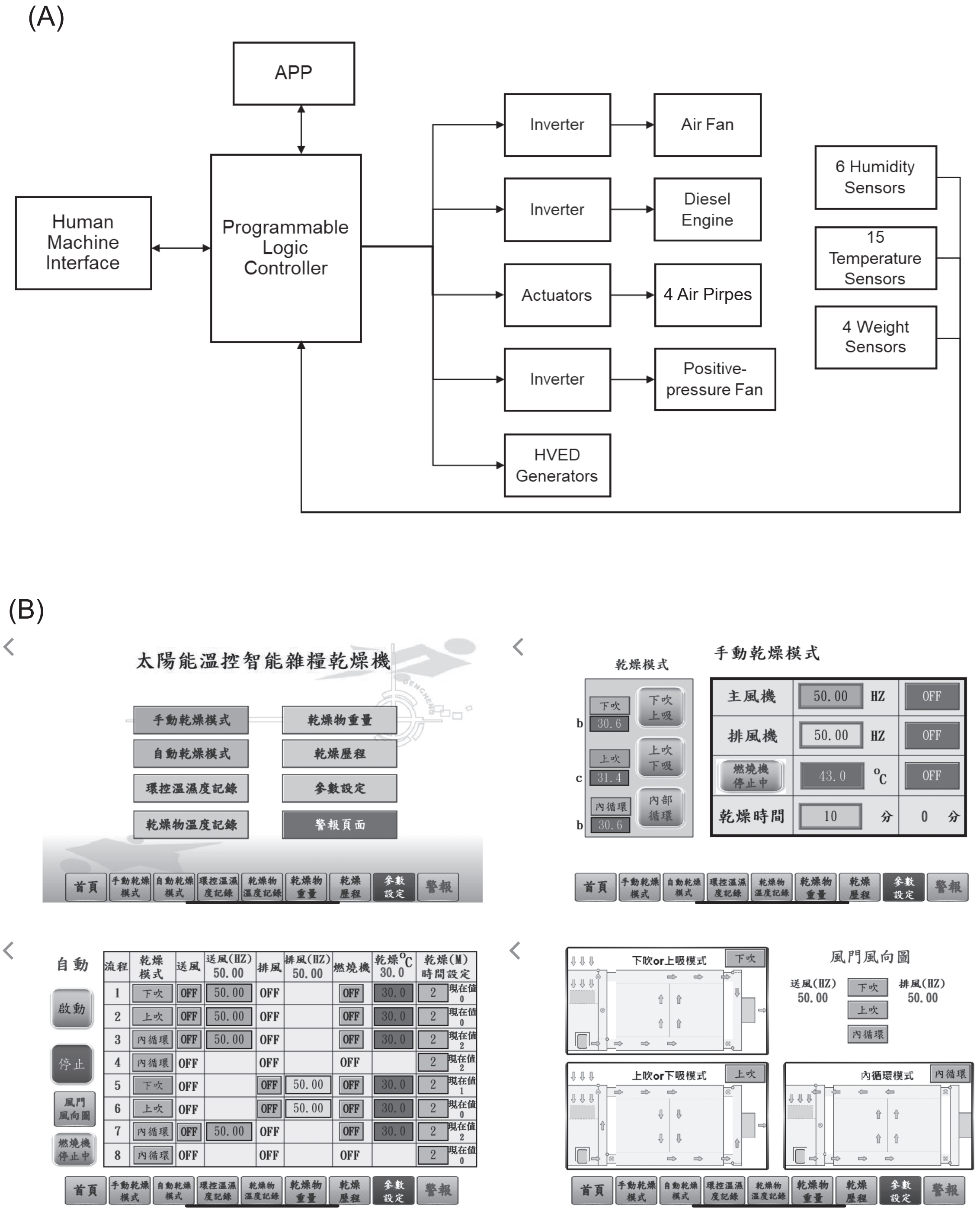
manually turned three times per day—at 10:00, 13:00, and 15:00—to ensure even drying. At 17:00 each day, the seeds were collected and stored in a shaded area at room temperature. The seed surface temperature was recorded daily at 13:00 to reflect the maximum thermal exposure during sun drying. Drying was performed for 8 h per day, except on weekends and days with unfavorable weather, when the seeds were stored indoors.

For the first batch, 3 kg of fresh camellia fruits (initial moisture content: 53.2%) harvested on October 26 were stored at 4℃ until the drying trial began. By the start of sun drying on December 13, the moisture content had naturally dropped to 32.9%. Drying was conducted from December 13 to 26, 2023, for a total of 9 drying days, excluding December 16–17 and 23–24 (weekends), and December 22 (a cloudy, cold day). Sun drying was terminated when the seed moisture content reached 10%. The average midday seed surface temperature during the drying period was 42.0℃.

For the second batch, 3 kg of fresh camellia fruits (initial moisture content: 47.8%) harvested on November 3 were similarly stored at 4℃. By the time sun drying began on January 2, 2024, the moisture content had decreased to 32.9%. Drying continued until January 12, requiring an additional 9 drying days. Drying was not performed on January 6–7 and 13–14 (weekends), during which the seeds were stored indoors. The drying process was terminated when the seed moisture content reached 10%. The average midday seed surface temperature was 46.6℃.

Moisture content determination

The moisture content of camellia seeds was determined using a moisture analyzer (Model MX-50, A&D Company, Limited, Tokyo, Japan). A single dehulled seed was ground into a fine powder using a laboratory grinder. Approximately 0.5 g of the ground sample was evenly spread on the sample pan of the analyzer. The measurement was performed at 105℃ using the automatic end-point detection mode according to the manufacturer's instructions. Each mea-


(A)
APP
Human Machine Interface
Programmable Logic Controller
Inverter
Air Fan
Inverter
Diesel Engine
Actuators
4 Air Pirpes
Inverter
Positive-pressure Fan
HVED Generators
6 Humidity Sensors
15 Temperature Sensors
4 Weight Sensors
(B)
太陽能溫控智能雜糧乾燥機
手動乾燥模式
自動乾燥模式
環控溫濕度記錄
乾燥物溫度記錄
乾燥物重量
乾燥歷程
參數設定
警報頁面
乾燥模式
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主風機
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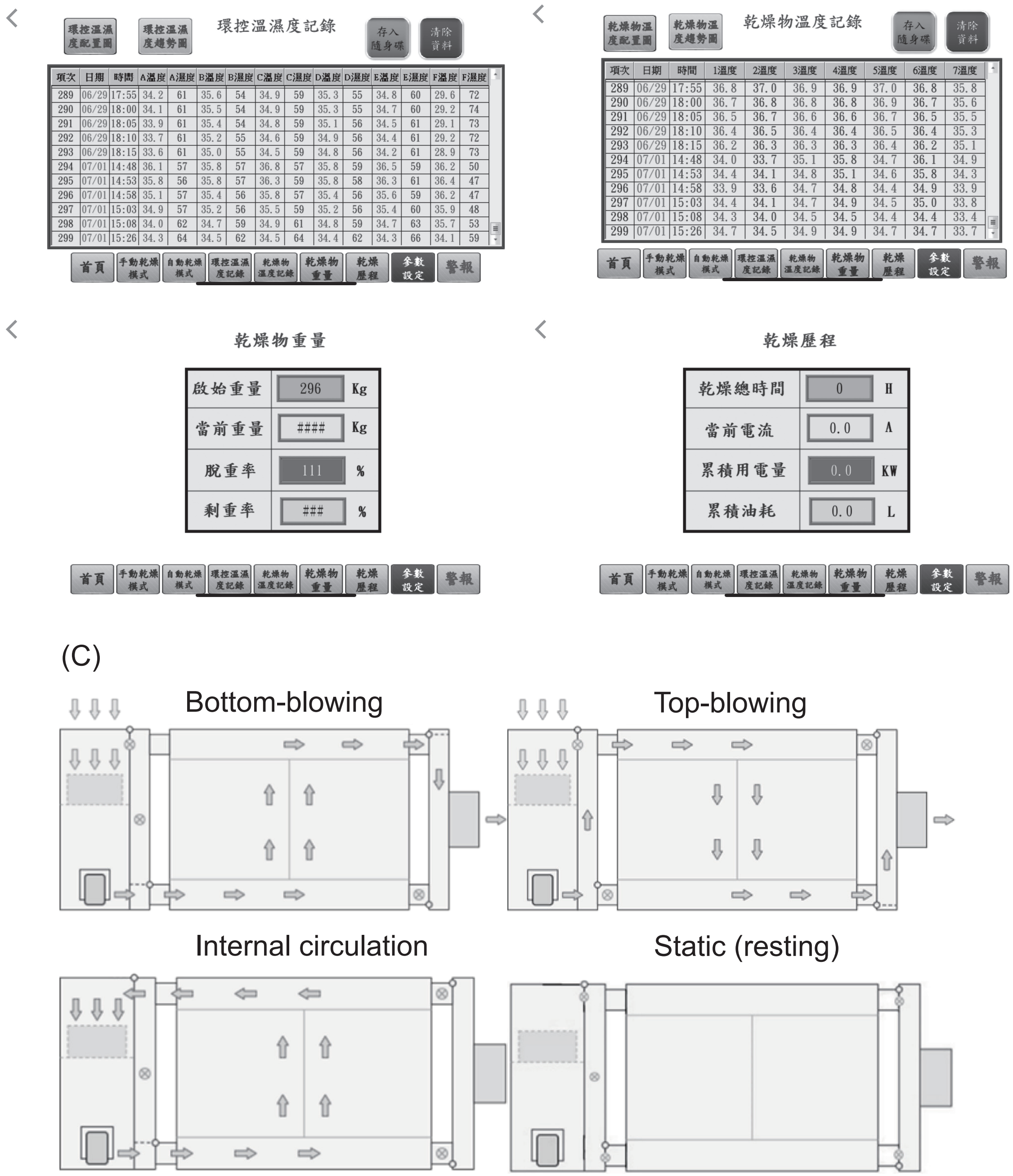

Fig. 3. Intelligent monitoring and control system of the electrohydrodynamic (EHD) dryer. (A) System flowchart of the human-machine interface (HMI) control panel with real-time monitoring functions. (B) Layout of temperature and humidity control points inside the drying chamber (interface display in Chinese). (C) Sensor layout and schematic diagram of internal circulation airflow modes within the chamber, illustrating integration of temperature, humidity, and weight sensors for precision control. HVED: high voltage electrical discharge.

surement was conducted in triplicate, and the average moisture content was calculated and expressed as a percentage on a wet basis.

Oil extraction and quality evaluation

For oil extraction, unroasted camellia seeds were used and pressed with a screw-type oil expeller (Model: SX-TB05, Orin Oil Press, Style International Development Co., Ltd., New Taipei, Taiwan). The obtained crude oil was subjected to centrifugation to remove sediment, and the clear upper oil layer was collected for further analyses. The evaluation indicators included drying time (defined as the time required to reduce the moisture content below 10%), energy consumption (expressed in kWh per kilogram of dried material), oil quality parameters- specifically acid value (AV) and peroxide value (POV)- as well as oxidative stability, measured by the oil stability index (OSI) (Lee *et al*. 2023). AV and POV values are presented as mean ± standard deviation (n = 2 for AV, n = 4 for POV). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Comparison of drying efficiency between the intelligent EHD dryer and traditional sun drying

To evaluate the effectiveness of the intelligent EHD dryer, its drying performance was compared with traditional sun drying, using camellia fruits as the model material. Key aspects assessed included labor intensity, operational control, and drying efficiency. As summarized in Table 3, the intelligent EHD system exhibited clear advantages in automation, moisture control, and uniformity, while substantially reducing manual labor.

In this study, 90 kg of small-seed camellia fruits (seed diameter: 1–2 cm) were processed in two batches using the intelligent EHD dryer (Fig. 4). Initial seed moisture contents were 51.2% (Batch 1) and 47.3% (Batch 2). The drying process was divided into two stages. The first 24 h employed alternating top-down and bottom-up hot-air flow at 42℃ to promote hull cracking. By the end of this phase, hulls were manually removed, and seed moisture had dropped to

Table 3. Comparison of advantages and disadvantages of different drying methods.

Criteria	Traditional sun drying	Conventional cabinet dryer	Intelligent electrohydrodynamic (EHD) dryer
Weather dependence	High	Not affected	Low
Space requirement	High	High	Low
Labor requirement	High	High	Low
Drying process	Manual turning	Manual turning	No turning required
Drying uniformity	Easily uneven	Easily uneven	High
Material loss during process	Likely	Likely	None
Moisture content monitoring	Manual	Manual	Automated
External energy requirement	None	Required	Required
Loss rate	High	High	Low
Carbon footprint	Low	High	Low
CO_2 emissions	None	High	Low
Drying cost	Medium	High	Low

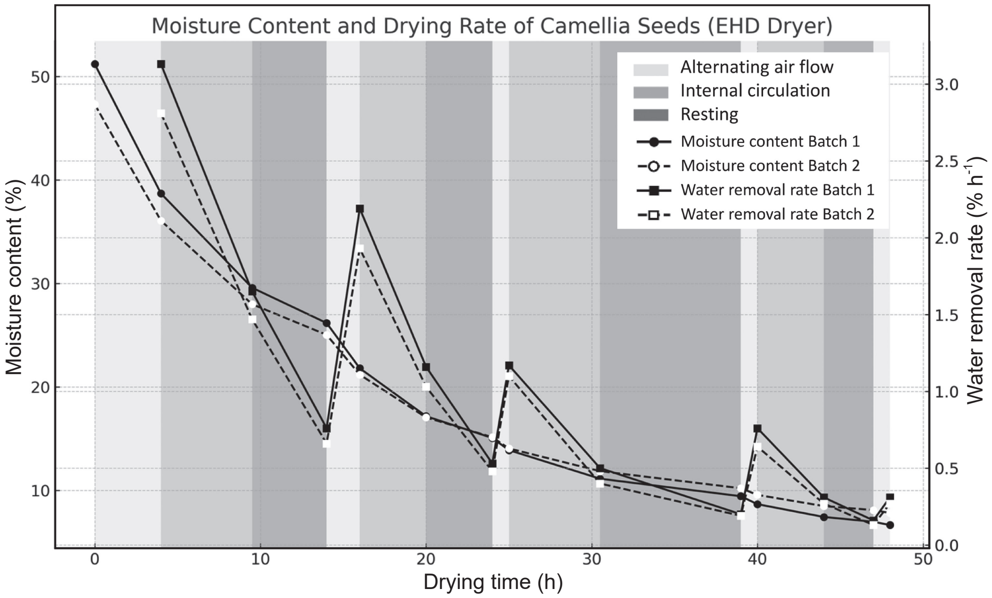

Fig. 4. Drying curves and water removal rates of two batches of camellia seeds using the intelligent electrohydrodynamic (EHD) dryer at 42℃ over 48 h. The drying process alternated between three operational modes- alternating top and bottom airflow with opposite suction, internal circulation, and resting- which are represented by distinct gray-shaded regions. The moisture content of batch 1 decreased from 51.2% to 6.7%, and that of batch 2 decreased from 47.3% to 7.8%. Solid and dashed lines represent the drying profiles of Batch 1 and Batch 2, respectively. Drying rate (% h^{-1}) was calculated for each interval and plotted at the end point of the corresponding drying period. The curves illustrate a typical three-phase drying pattern without an initial lag phase, highlighting reproducibility and consistent drying kinetics under identical EHD conditions.

15.1–11.5%. In the second stage, the system switched to internal circulation with intermittent resting (2 h every 6 h), resuming drying for another 24 h until seed moisture reached 6–8%. Thus, the entire EHD drying process achieved stable dehydration within 48 h, suitable for storage and oil pressing.

Unlike traditional cabinet dryers, which require frequent manual stirring to avoid uneven drying, especially for large-volume materials like camellia seeds, the intelligent EHD dryer eliminates this need. Its programmable airflow-switching system—capable of alternating between vertical airflow and recirculating modes—allowed the seeds to remain static throughout the drying period while ensuring homogenous moisture reduction. Temperature and humidity were well-controlled throughout the process, with monitored values of 41.0 ± 1.1℃ and ± 3% RH variation across six probes and seven embedded seed sensors (Fig. 2B).

The drying curves of the two batches (Fig. 4) demonstrated reproducible behavior under identical conditions. Moisture content declined from 51.2% to 6.7% (Batch 1) and from 47.3% to 7.8% (Batch 2). Three drying phases were observed: a rapid initial drop (0–10 h), a falling-rate phase (10–30 h), and a gradual plateau (30–48 h), consistent with classical drying models. Notably, no evident lag phase was present at the beginning, suggesting that ion wind-assisted airflow in the EHD system effectively removed surface moisture from the outset.

To further quantify drying performance, water removal rate (% h^{-1}) was calculated and plotted against time for both batches (Fig. 4). Both curves showed a sharp initial drying rate followed by a gradual decline, consistent with moisture diffusion limitations as drying progressed. The peak drying rate occurred within

the first 2–6 h and reached over 3% h^{-1} for Batch 1 and approximately 2.7% h^{-1} for Batch 2. This pattern aligns well with the observed three-phase drying behavior. Moreover, the drying rate curves revealed minimal variation between batches, confirming consistent system performance and effective airflow management.

The close overlap between the drying curves and drying rate profiles confirms strong batch-to-batch reproducibility, despite minor initial moisture differences. This highlights the robustness of the system's intelligent control, which adapts airflow modes and tempering schedules in real time. Together, these findings validate the practical value of the intelligent EHD dryer for scalable agricultural drying with minimal variability and labor requirements.

Drying behavior of camellia seeds under intelligent EHD dryer

In this study, the drying process of camellia seeds using an intelligent EHD dryer exhibited four distinct cyclic stages, as shown in the moisture content and drying rate curves. Each cycle consisted of three operational phases: directional blowing (either bottom-up or top-down), internal circulation, and a resting period. Notably, the so-called bottom-up and top-down blowing phases involved a paired airflow system with forced air injection from one end and simultaneous suction from the opposite end, i.e., bottom-blowing with top-suction, and vice versa. This design facilitated efficient and uniform moisture removal across the seedbed.

First cycle (0–14 h)

Bottom-up blowing phase (0–4 h): The initial moisture contents were 51.2% (Batch 1) and 47.3% (Batch 2). This phase showed the highest water removal rate (~3.1 % h^{-1}), as surface moisture rapidly evaporated and exited the drying chamber.

Internal circulation phase (4–9 h): With the chamber sealed, vapor could not escape. Moisture within the material is redistributed toward the surface, resulting in a more uniform internal moisture distribution. Although the external moisture removal rate decreased slightly, internal redistribution was significantly enhanced.

Resting phase (9–14 h): A sharp drop in drying rate was observed. At this stage, the surface was relatively dry and hot, while the interior remained moist and cool. Heat is transferred inward, energizing internal water molecules and facilitating their outward diffusion.

Second cycle (14–24 h)

Top-down blowing phase (14–16 h): Reversing the airflow direction (now top-blowing with bottom-suction) eliminated the need for manual turning. Moisture removal accelerated again, with moisture content dropping from ~25% to ~17%.

Internal circulation phase (16–20 h): A slight decline in drying rate occurred, but internal moisture continued to migrate outward, promoting further redistribution.

Resting phase (20–24 h): The drying rate decreased noticeably again, as moisture continued diffusing from the core to the surface.

Subsequent cycles (24–48 h)

The alternating airflow and resting strategy effectively prevented case hardening, a phenomenon where overly dried surfaces hinder internal moisture transfer. This drying protocol maintained a continuous moisture gradient, ensuring connectivity between internal and external moisture phases, and thereby sustaining water migration. By the end of 48 h, final moisture contents reached 6.7% and 7.8% for Batch 1 and Batch 2, respectively.

Compared to conventional drying methods applied to oil-rich seeds such as *C. oleifera*, the intelligent EHD drying system demonstrates distinct advantages in both drying kinetics and operational efficiency. The hot-air drying curve of *C. oleifera* seeds consists of a very short acceleration rate period at the beginning and a long falling rate period, indicating that the drying of *C. oleifera* seeds is mainly controlled

by the diffusion of moisture inside the material (Huang *et al*. 2020). This underscores a critical challenge in drying oil-rich seeds: the removal of internal moisture is often hindered by the dense seed structure and high lipid content, which can lead to case hardening and uneven dehydration if not properly managed.

In response to this challenge, Zhang *et al*. (2023) developed a microwave-vacuum drying system specifically designed to enhance internal moisture migration. By combining volumetric heating with reduced pressure, their method accelerated water diffusion from the core to the surface, significantly shortening drying time. However, the study also reported that excessive microwave power could cause seed shell hardening and rupture, ultimately impeding moisture escape and compromising oil quality. Additionally, the system's sensitivity to loading quantity and power settings suggests that precise control is necessary to avoid thermal damage.

In contrast, the intelligent EHD drying system developed in this study offers a non-thermal solution to the same problem. Operating at a low temperature of 42℃, the EHD dryer employs cyclic airflow modes- including directional ion wind, internal circulation, and resting phases- to sustain moisture migration without inducing surface hardening. This approach effectively maintains a continuous moisture gradient, allowing internal water to diffuse outward even under high loading conditions. As a result, the EHD system achieves uniform drying while preserving seed integrity and oil quality, demonstrating its suitability for oil-rich seeds that are prone to diffusion-limited dehydration.

Comparison of pressed oil quality from camellia seeds

To assess the impact of drying methods on oil quality, camellia seeds were dried using either traditional sun drying or the EHD drying method at 42℃ and then analyzed. After hull removal, the kernels were mechanically pressed using a screw-type oil expeller to obtain crude oil. The extracted oils were subsequently analyzed for acid value (AV), peroxide value (POV), and OSI, which are key indicators of oil degradation and oxidative stability.

As shown in Fig. 5, the AVs of oils from two batches of EHD-dried seeds were 0.44 and 0.33 mg KOH g^{-1} oil, respectively, which were significantly lower than those of oils from sun-dried seeds (0.78 and 0.81 mg KOH g^{-1} oil). This suggests that the EHD drying process might be more effective in inhibiting free fatty acid formation. Similarly, the POVs were notably lower in the EHD-dried samples (both 0.25 meq O_2 mL^{-1} oil), compared to 0.99 and 0.49 meq O_2 mL^{-1} oil for sun-dried samples. These results suggest that EHD drying might lead to lower initial oxidation and better oil freshness.

Oxidative stability was further evaluated using the OSI, which measures the induction time (in hours) before rapid oxidation occurs under accelerated conditions. OSI was tested at 110℃, 120℃, and 130℃ with 10 L h^{-1} of air injected into the reaction chamber. As shown in Fig. 6 and Table 4, the OSI values decreased by approximately half for every 10℃ increase in temperature, consistent with a Q_{10}-type thermal oxidation reaction, indicating typical chemical degradation kinetics. The OSI values of oils from EHD-dried and sun-dried seeds were comparable across temperature settings.

Interestingly, a notable difference was observed between the two batches despite only a one-week difference in harvest timing. In the first batch, OSI values at 110℃, 120℃, and 130℃ were approximately 8, 4, and 2 h, respectively. In contrast, the second batch showed higher stability, with OSI values of approximately 12, 6, and 3 h at the same test temperatures. These results suggest that seed maturity at harvest may influence oil quality, with more mature seeds providing higher oxidative stability and oil content (Luo *et al*. 2012). These results suggest that seed maturity at harvest plays a critical role in determining oil quality, with more mature seeds providing higher oxidative stability and oil content. As reported by Luo *et*

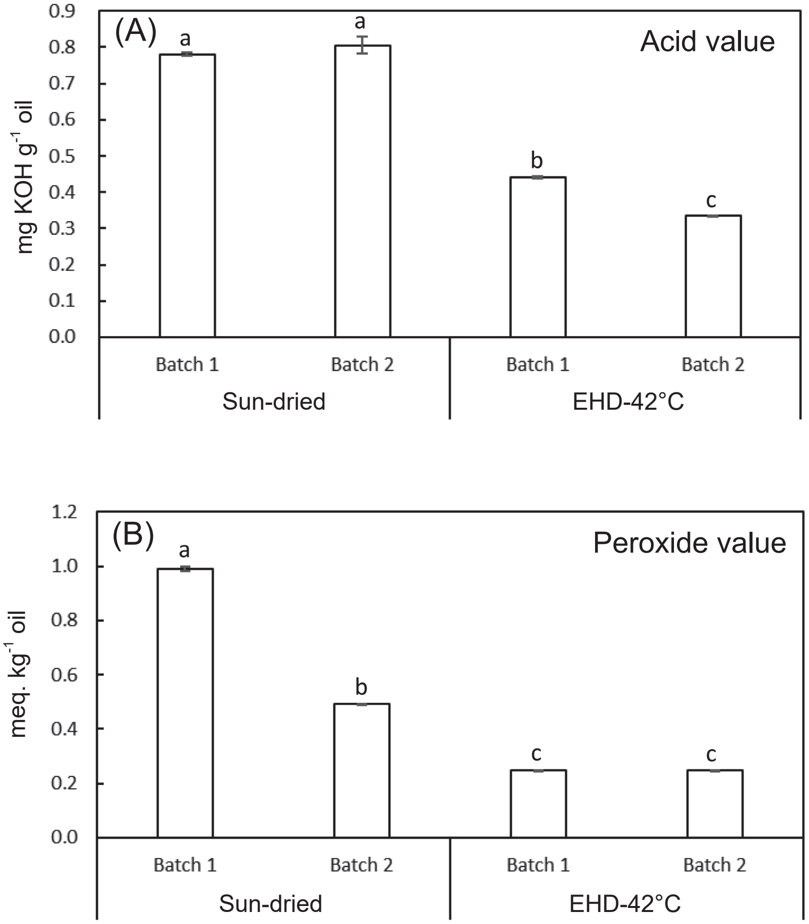

Fig. 5. Acid and peroxide values of oils from camellia seeds dried by sun drying and electrohydrodynamic (EHD) drying. (A) Acid value (AV) was analyzed with duplication; (B) Peroxide value (POV) was conducted with tetraplication. One-way analysis of variance (ANOVA) with Tukey's multiple comparison test (significant $P < 0.05$) was conducted. AV and POV determinations were mean and standard deviation ($n = 2$ and 4, respectively) are shown on the column, and different letters indicate statistically significant differences.

al. (2012), the contents of unsaturated fatty acids, vitamin E, and β-sitosterol in camellia oil increased significantly as the seeds matured, particularly between October 9 and 24, reaching a peak around October 29. Furthermore, oil extracted from naturally fallen fruits exhibited the highest acid and peroxide values, indicating that over-mature fruits might be prone to hydrolytic and oxidative degradation. Delayed harvesting also reduced spicy and astringent flavors in the oil, while increasing the content of saponins in the press cake. These findings support the importance of optimal harvest timing- just before natural abscission- to ensure maximum oil quality and retention of bioactive compounds.

Sensory evaluations revealed subtle differences in flavor profiles. In the first batch, oils from EHD-dried seeds exhibited a buttery sweetness with mild pungency, whereas oils from sun-dried seeds had a slightly bitter note, moderate sweetness, and a stronger aroma. In the second batch, both drying methods produced oils with a clean, sweet, and fruity char-

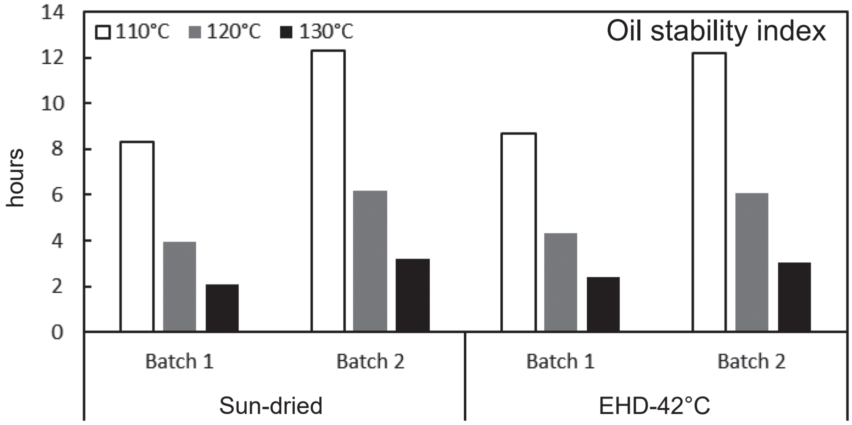

Fig. 6. Oil stability index (OSI) of camellia oil at different oxidation temperatures. OSI values (in hours) of oils extracted from camellia seeds dried by sun drying and EHD drying at 42℃, measured at 110℃, 120℃, and 130℃ under accelerated oxidation conditions. A typical Q_{10}-type degradation pattern was observed. While OSI values were comparable between drying methods, noticeable differences were observed between batches, underscoring the influence of harvest timing on oxidative stability.

Table 4. Oil stability indexes (OSI) and predicted oil stable times at frying temperatures.

		OSI			Prediction[z]	
Drying method	Batch	110℃ (h)	120℃ (h)	130℃ (h)	160℃ (min)	180℃ (min)
Sun dried	1	8.1	4.1	2.0	15.4	3.9
	2	12.2	6.2	3.2	25.1	6.5
EHD at 42℃	1	8.5	4.5	2.3	20.1	5.5
	2	12.2	6.1	3.1	23.0	5.8

[z] Prediction with extrapolation method from OSI values at 110, 120 and 130℃.

acter and no detectable bitterness, suggesting that harvest timing might influence both chemical and sensory attributes.

CONCLUSION

This study evaluated the application of EHD drying for camellia oil seed dehydration and compared its performance with traditional sun drying. Key parameters assessed included drying efficiency, labor intensity, oil quality, oxidative stability, and sensory characteristics of the extracted oil. Results demonstrated that the intelligent EHD dryer significantly improved drying uniformity, reduced drying time, and eliminated the need for manual turning, offering a fully automated and weather-independent solution.

In addition, EHD drying preserved oil quality more effectively, producing oils with lower acid and peroxide values than sun-dried samples, indicating reduced hydrolysis and oxidation. Operating at lower temperatures, EHD drying also minimized thermal degradation, thereby enhancing the retention of bioactive compounds and oxidative stability.

These findings support the feasibility of EHD technology as a novel, non-thermal drying method for camellia seeds and suggest its broader applicability to other agricultural products. Future research should explore optimal combinations of electric field intensity and temperature to further enhance drying performance. The potential to extend this approach to other oil-rich seeds, such as peanuts, sesame, and soybeans, as well as its integration

with different drying methods like freeze or hot-air drying, warrants further investigation.

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智慧型電流體動力乾燥機於茶油籽乾燥效率提升之應用

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摘要

曾祥恩、李雅琳、李育賢、林佫群、蘇文瑞。2025。智慧型電流體動力乾燥機於茶油籽乾燥效率提升之應用。台灣農業研究 74(4):541–556。

本研究應用電流體動力 (electrohydrodynamic; EHD) 乾燥技術，使用自行研發之智能型電流體乾燥機，乾燥臺灣原生種短柱山茶 (*Camellia brevistyla*) —— 小果油茶籽，以與傳統日曬法進行比較。評估項目包含油茶籽乾燥效率、冷壓油脂之品質 (酸價 (acid value; AV) 與過氧化價 (peroxide value; POV)) 以及油脂氧化安定性 (oil stability index; OSI)。試驗材料採用 2 批次油茶籽，EHD 乾燥之環境溫度設定於 42℃，此溫度為日曬法可以達到的一般平均高溫。試驗結果顯示，智能型電流體乾燥技術能顯著提升乾燥效率與縮短乾燥時間 (48 h)。相對地，日曬法需 9 d 以完成乾燥。此智能型電流體乾燥方法可以均勻乾燥與減少人工翻堆需求。油脂品質分析結果顯示，電流體乾燥處理的油茶籽壓榨油，其酸價與過氧化值均顯著低於傳統日曬法，顯示其壓榨油品質更佳；油脂氧化安定性 (OSI) 與官能品評之風味特徵則兩種乾燥法相近。綜合評估，電流體乾燥技術應用於油茶籽乾燥，可以提升乾燥效率與其壓榨油之油脂品質，因此具有商業化的應用潛力。

關鍵詞：短柱山茶、油茶籽、油茶籽乾燥、電流體乾燥、油脂品質。

投稿日期：2025 年 5 月 27 日；接受日期：2025 年 9 月 12 日。

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(原《中華農業研究》期刊)

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(Formerly Journal of Agricultural Research of China)

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Reviewers, Journal of Taiwan Agricultural Research
Volume 74, 2025

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JOURNAL OF TAIWAN AGRICULTURAL RESEARCH

Vol. 74 No. 4 December 31, 2025

Published by Taiwan Agricultural Research Institute, Ministry of Agriculture
Publisher Dennis Wang
Editor-in-Chief Hsien-Tzung Shih
Address No.189, Zhongzheng Rd., Wufeng Dist., Taichung City 413008, Taiwan, ROC
Website https://www.tari.gov.tw/jtar; E-mail: jtar@tari.gov.tw; Tel: +886-4-2331-7236
Submission https://www.ipress.tw/J0042
Complete instructions to authors are available on Journal of Taiwan Agricultural Research website: https://www.tari.gov.tw/jtar/

Press Ainosco Press
Address 18F, No.80, Sec.1, Chenggong Rd., Yonghe Dist., New Taipei City 234634, Taiwan, ROC
Tel +886-2-2926-6006
E-mail press@airiti.com

Price NT$ 175 (per copy)
Distributor 1. GB Books / 1F., No.209, Songjiang Rd., Taipei City 104472, Taiwan, ROC
GB Website: http://www.govbooks.com.tw; Tel: +886-2-2518-0207
2. Wunan Books / No.6, Zhongshan Rd., Taichung City 400002, Taiwan, ROC; Tel: +886-4-2226-0330